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Programa de Doctorado en Biociencias Moleculares

Estudio de la función y potencial pronóstico de las proteínas de unión a RNA, PIWI y UNR, en cáncer de páncreas

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Memoria presentada por Dña. Weiyao Li

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Certifican:

que Weiyao Li ha realizado, bajo nuestra dirección, el trabajo titulado

¨Estudio de la función y potencial pronóstico de las proteínas de unión a RNA, PIWI y UNR, en cáncer de páncreas

Para optar al grado de Doctor en Biociencias Moleculares.

Dicho trabajo reúne, a nuestro juicio, las condiciones de originalidad y rigor necesario y, por tanto, avalamos su presentación para ser juzgado.

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“Confía en el tiempo, que suele dar dulces salidas a muchas amargas dificultades”

Miguel de Cervantes Saavedra

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RESUMEN

El cáncer de páncreas es una manifestación altamente agresiva del cáncer y presenta resultados clínicos pobres debido a su diagnóstico tardío con enfermedad metastásica. Actualmente, faltan biomarcadores tanto de pronóstico como predictivos de respuesta a tratamientos. Las proteínas de unión a ARN (RBP, del inglés *RNA Binding Proteins*) juegan un papel vital en la progresión de muchos tipos de cáncer. Este proyecto de investigación se centra en dos RBP humanas, PIWI y UNR, que actúan a nivel de ARN pequeños no codificantes (piRNAs) y a nivel de ARN mensajero (ARNm), respectivamente. La expresión de las proteínas PIWI (*P-element-induced wimpy testis)* promueve algunas de las características del cáncer, como la proliferación celular, la integridad genómica, la apoptosis, la invasión y la metástasis. Otra proteína de unión a ARN llamada *Upstream of N-Ras* (UNR), codificada por el gen *CSDE1* (*Cold Shock Domain Containing E1*) se encuentra próximo al extremo 5´UTR del locus de *NRAS*, y se ha demostrado que regula los ARN mensajeros de c-Fos, c-Myc, Pten, Rac1 o Vimentin. El objetivo de mi Tesis Doctoral es esclarecer la función y el potencial pronóstico de estas proteínas de unión a ARN, PIWI y UNR, en el cáncer de páncreas.

La Tesis Doctoral se presenta como un compendio de publicaciones, la primera de las cuales muestra que la expresión de PIWIL2 se asoció significativamente con una mayor supervivencia libre de progresión y supervivencia global en pacientes con cáncer de páncreas. También, revelamos que PIWIL1 y PIWIL2, tanto en los niveles de expresión de ARNm como de proteína, se correlacionaron positivamente con factores asociados al subtipo molecular progenitor de cáncer pancreático. En el segundo artículo mostramos una expresión diferencial en líneas celulares tumorales y no tumorales de PIWIL3 y PIWIL4. Luego, realizamos experimentos funcionales, los resultados apuntan a PIWIL3 y PIWIL4 como factores cruciales en la regulación de la motilidad celular, el mantenimiento de las células desdiferenciadas y la resistencia a quimioterapias tanto en las células tumorales como en las células pancreáticas sanas. Además, la baja expresión de PIWIL4 es capaz de predecir una supervivencia más corta de los pacientes con cáncer de páncreas. En el tercer trabajo, mostramos que la baja expresión de UNR indica un mal pronóstico de pacientes con cáncer de páncreas. Además, la expresión UNR se asoció con el subtipo molecular inmunogénico del cáncer de páncreas. En base a estos hallazgos, proponemos UNR como un biomarcador de pronóstico para el cáncer de páncreas .

ABSTRACT

Pancreatic cancer is a highly aggressive manifestation of cancer and presents poor clinical results due to its late diagnosis with metastatic disease. Currently, biomarkers lack both prognosis and predictive response to treatment. RNA binding proteins (RBPs) play a vital role in the progression of many types of cancer. This research project focuses on two human RBPs, PIWI and UNR, which act at the level of small non-coding RNA (piRNAs) and at the level of messenger RNA (mRNA), respectively. Expressions of PIWI (P-element-induced weakened testis) proteins promote some of the characteristics of cancer, such as cell proliferation, genomic integrity, apoptosis, invasion, and metastasis. Another RNA-binding protein called N-Ras Upstream (UNR), encoded by the CSDE1 gene (Cold Shock Domain Containing E1) is found near the 5 'UTR end of the NRAS locus, and has been found to regulate messenger RNAs of c-Fos, c-Myc, Pten, Rac1 or Vimentin. The objective of my Doctoral Thesis is to clarify the function and prognostic potential of these RNA-binding proteins, PIWI and UNR, in pancreatic cancer.

The Doctoral Thesis is presented as a compendium of publications, the first of which showing PIWIL2 expression was associated with increased progression-free survival and overall survival in patients with pancreatic cancer. Also, we reveal that PIWIL1 and PIWIL2, both in mRNA and protein expression levels, are positively correlated with factors associated with the progenitor molecular subtype of pancreatic cancer. In the second article, we show differential expression in tumor and non-tumor cell lines of PIWIL3 and PIWIL4. Then, we carried out functional experiments, the result point to PIWIL3 and PIWIL4 as crucial factors in the regulation of cell motility, the maintenance of dedifferentiated cells and resistance to chemotherapies in both tumor cells and healthy pancreatic cells. Furthermore, the low expression of PIWIL4 is capable of predicting a shorter survival of patients with pancreatic cancer. In the third work, we show that the low expression of UNR indicates a poor prognosis in patients with pancreatic cancer. Furthermore, UNR expression is associated with the immunogenic molecular subtype of pancreatic cancer. Based on these findings, we propose UNR as a prognostic biomarker for pancreatic cancer.

ÍNDICE

ABREVIATURAS

CaPa: Cáncer de páncreas

PIWI: P-element-induced wimpy testis

AGO: familia de proteínas argonautas

piRNAs: RNA que interactúan con PIWI

piRISC: complejo silenciador inducido por piRNA

CpG: regiones de ADN ricas en nucleótidos de citosina y guanina

TERT: transcriptasa inversa de telomerasa

WB: Western Blot

IHC: inmunohistoquímica

siRNA: RNA de interferencia corta

TEM: transición epitelial a mesenquimal

FOLFIRINOX: 5-fluorouracilo, leucovorina, irinotecán y oxaliplatino

Nab-Paclitaxel: Paclitaxel unido a nanoalbúmina

HNF4A: factor nuclear de hepatocitos 4 alfa

TCGA: El Atlas del Genoma del Cáncer

FDA: Administración de Alimentos y Medicamentos

ARNm:Ácido Ribonucleido mensajero

TAC:Tomografía Axial Computarizada

RMN:Resonancia Magnética Nuclear

RBPs: las proteínas de unión a RNA

URN: Upstream of N-ras

CPRE: colangiopancreatografía retrógrada endoscópica

PAAF: aspiración con aguja fina

RT: radioterapia

ER: estado de rendimiento

LV: leucovorina

5-FU: 5-fluorouracil

LSN:límite superior de lo normal

AUB: Protein aubergine

AGO3: Protein argonaute-3

CCR: cáncer colorrectal

CHC: Carcinoma hepatocelular

CDK2: Cyclin-dependent kinase 2

OIP5-AS1:OIP5 ARN antisentido 1

JAK2: Janus kinase 2

TGF-β: Factor de crecimiento transformante beta

MAPK: Proteína quinasa activada por mitógeno

ERK : Quinasas reguladas por señal extracelular

FGF: Factor de crecimiento de fibroblastos

INTRODUCCIÓN

1.CÁNCER DE PÁNCREAS

El cáncer de páncreas (CaPa) es un tumor maligno cuyo pronóstico general es bastante sombrío, y se ha mantenido prácticamente sin cambios durante muchas décadas. Es el décimo tumor en frecuencia en los países industrializados, y constituye del 2% al 3% de todos los tumores sólidos(1). En Estados Unidos es el cuarto tumor más común(2), y representa la cuarta causa de muerte por cáncer en ambos sexos. Además, se prevé que para 2030 se incremente su incidencia en un 50%, convirtiéndose así en la segunda causa de muerte por cáncer y provocando más muertes que el cáncer de próstata, colon o mama(2).

Aunque la tasa de supervivencia a cinco años es del 50% cuando los tumores tienen un tamaño <2 cm y cerca del 100% para los tumores <1 cm(3). El CaPa sigue siendo una de las neoplasias más agresivas debido a su pronta difusión y su falta de síntomas específicos tempranos lleva a un diagnóstico tardío lo que impide la cirugía con intención curativa.

El CaPa se divide principalmente en dos tipos: el adenocarcinoma de páncreas, que es el más común representando 85% de los casos, surge en las glándulas exocrinas del páncreas, y el tumor neuroendocrino pancreático, que supone menos de un 5% y ocurre en el tejido endocrino del páncreas(4). El adenocarcinoma de páncreas tiene muy mal pronóstico en comparación con el tumor neuroendocrino pancreático, generalmente después del diagnóstico, sólo el 24% de las personas sobrevive 1 año y sólo el 9% vive durante 5 años(5).

1.1 Epidemiología

Tomando EE.UU como país de referencia, se estima que a lo largo de 2020 se diagnosticará CaPa a alrededor de 57.600 nuevos casos (30.400 casos en hombres y 27.200 en mujeres)(2). Las tasas de incidencia son un 25 % más altas en las personas de raza negra que en las personas de raza blanca. Y la incidencia es ligeramente superior en hombres que en mujeres con una proporción de 1,3:1 (Figura 1)(2).

Figura 1. El gráfico de barras muestra las tasas de incidencia y mortalidad para el CaPa para todas las edades en 2018 según las áreas mundiales y el sexo (reproducido de http://globocan.iarc.fr/).

Se estima que este año se producirán 47.050 muertes en los EE.UU (24.640 en hombres y 22.410 en mujeres) a causa de esta enfermedad(2). Además, la mayoría de pacientes a los que se les diagnostica un CaPa tienen una edad comprendida entre los 65 y los 70 años(6).

1.2 Etiología y factores de riesgo

No se ha definido una etiología clara del CaPa ni su mecanismo de carcinogénesis. Se cree que el proceso se inicia por una alteración de las células de los conductos pancreáticos. Los carcinógenos alcanzarían estas células provocando su transformación maligna por tres posibles vías de acceso: reflujo biliar, reflujo duodenal, o por vía sanguínea(4).

Hasta ahora, se han identificado varios factores de riesgo y se pueden dividir en dos categorías: factores de riesgos modificables y no modificables.

Los factores de riesgo modificables incluyen el tabaco(7), el alcohol, la obesidad, ciertos factores dietéticos y la exposición a otras sustancias tóxicas(8).

Los factores de riesgo no modificables incluyen la diabetes mellitus(9), los antecedentes familiares, la pancreatitis crónica, la infección por *H. Pylori*, la infección por el *Virus de la Hepatitis B* y otras afecciones hereditarias infrecuentes como(10): pancreatitis hereditaria, síndrome de Peutz-Jeghers, melanoma maligno familiar, síndrome de cáncer hereditario de mama y de ovario, síndrome de Lynch, síndrome de Li-Fraumeni o poliposis adenomatosa familiar.

1.3 Fisiopatología

En la mayoría de los tumores se encuentran alteraciones genéticas y/o cromosómicas.

1.3.1 Genética

La genética se ha convertido en un aspecto vital en la detección temprana del CaPa. Genes como *KRAS, CDKN2A, TP53 o SMAD4* se han relacionado con la mayoría de los casos(4). La comprensión de estos genes principales ha aportado una idea al diagnóstico y tratamiento del CaPa. Los principales genes impulsores del tumor pancreático presentan las siguientes tasas de mutaciones: *KRAS*(90%), *CDKN2A*(90%), *TP53*(70%), *SMAD4*(55%)(6).

Por otra parte, la vía Slit/Robo(5%), la ruta de señalización de Notch(5%), de WNT/Beta-Catenina(10%), SWI/SNF *chromatin-remodeling*(20%), la reparación del ADN(17%) o el ciclo celular(15%) son menores mecanismos implicados en el CaPa(11).

El gen *KRAS* es responsable del 90% de la mayoría de los casos de CaPa. La proteína RAS es responsable de la diferenciación y proliferación celular al enviar las señales para la diferenciación celular. La proteína RAS se une a GTP en el receptor G acoplado y da la señal de hidrólisis de GTP a GDP, lo que activa otras señales hacia abajo de la vía que llevan a una proliferación y crecimiento incontrolados. La mutación en el gen, hace que la proteína RAS resultante aumente la vida media del complejo RAS-GTP, y se prolonge las señales para una proliferación incontrolada(12).

TP53 es un gen supresor de tumores cuya proteína juega un papel importante en la regulación de la apoptosis al detener principalmente las células en la fase G1-S. Su inactivación por mutación puntual provoca varios cambios en el ciclo celular. Esto hace que se omitan varios puntos de control del ciclo celular, lo que induce nuevas mutaciones genéticas y, por lo tanto, el inicio del cáncer(13).

CDKN2A (que codifica para la proteína P16) es otro gen supresor de tumores que regula la fase G1-S del ciclo celular en un tumor pancreático. Cuando el gen *CDKN2A* se inactiva, conduce a un crecimiento y diferenciación incontrolados(13).

DPC4 es un gen supresor tumoral que codifica para la proteína SMAD4. Éste activa las uniones de TGF a los receptores de la superficie celular. Esto envía señales al núcleo para activar la transcripción del gen *DPC4*, para unirse a otras proteínas para regular y controlar el crecimiento y la proliferación. La mutación en este gen causa una proliferación y un crecimiento incontrolados asociados al CaPa(13).

1.3.2 Epigenética

La regulación epigenética de la expresión génica se produce a través de modificaciones covalentes en el ADN o en las histonas, que modifican el posicionamiento de nucleosomas y la acción de ARN no codificantes jugando un papel importante en el desarrollo del CaPa.

La metilación del ADN es uno de los mecanismos que inactiva los genes supresores. Estos genes no sufren ninguna mutación, pero los grupos metilo a nivel celular se agregan al carbono 5 del anillo de pirimidina que silencia el gen(11). Estudios recientes han demostrado que múltiples genes están silenciados o metilados en carcinomas pancreáticos. En este estudio se observó que *RARb, CDKN2A, CACNAIG, TIMP-3, ECAD, THBSI, HMLH1, DAPK1 y MINT31* son genes susceptibles de metilación en el CaPa(14). La sobreexpresión de EGF, EGFR, HER-2/NEU o P185 son eventos que se encuentran comúnmente en tumores pancreáticos de estadios avanzados(15).

También, se ha observado que algunos micro-ARN se desregulan en algunos adenocarcinomas pancreáticos. Por ejemplo, miR-21 se ha encontrado sobreexpresado en tejidos de carcinoma pancreático y líneas celulares en comparación con tejidos normales(15).

1.4 Estadiaje del cáncer de páncreas

Para la confirmación del diagnóstico se realiza la Tomografía Axial Computarizada (TAC) o la Resonancia Magnética Nuclear (RMN) abdominal(16). Otras técnicas como la colangiopancreatografía retrógrada endoscópica (CPRE) y la ecoendoscopia, permiten obtener muestra para el diagnóstico citológico y genético. El diagnóstico requiere la confirmación histológica o citológica, a través de muestras obtenidas por punción aspiración con aguja fina (PAAF) dirigida por ecografía o ecoendoscopia, o bien por cepillado/aspiración del jugo pancreático mediante CPRE (Figura 2)(6).

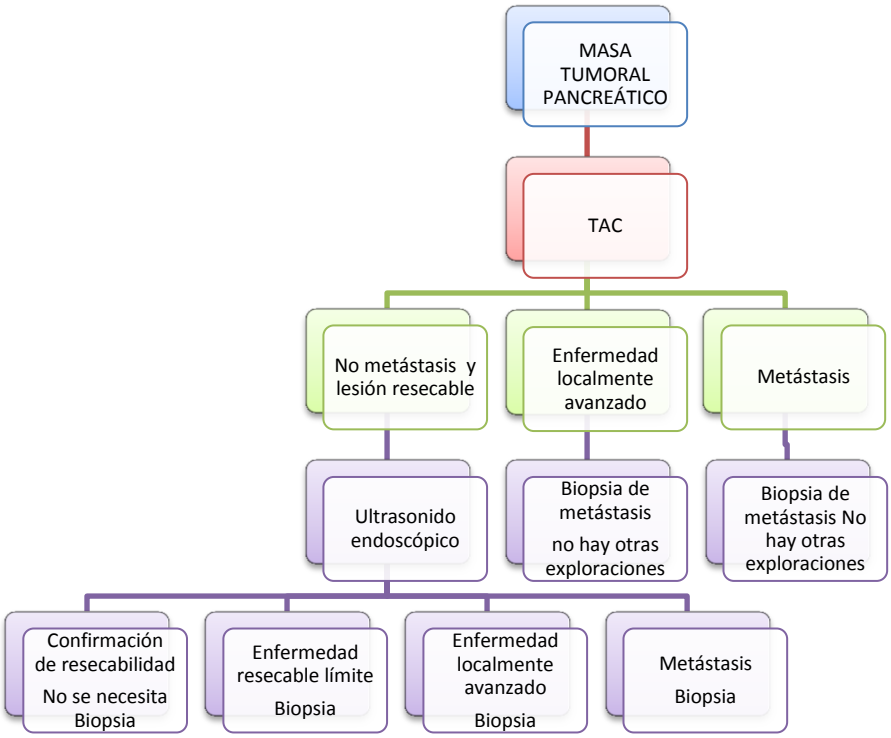

Figura 2. Análisis diagnóstico previo a la decisión multidisciplinaria. (Fuente: adaptado ESMO Clinical Practice Guidelines 2019)

El sistema de estadificación que se emplea con más frecuencia para el CaPa es el sistema TNM de la *American Joint Committee on Cancer* (AJCC) (Tabla 1). Se utiliza para la mayoría de los cánceres pancreáticos, excepto para los tumores neuroendocrinos bien diferenciados, los cuales tienen su propio sistema de estadificación.

Tabla 1. Classificación TNM y estadificación del CaPa.

Table 1. TNM classification 7th edition
Primary tumour (T)
T0 = No evidence of primary tumour
Tis = Carcinoma in situ
T1 = Tumour limited to the pancreas, ≤2 cm in greatest dimension
T2 = Tumour limited to the pancreas, >2 cm in greatest dimension
T3 = Tumour extends beyond the pancreas but without involvement of the coeliac axis or the superior mesenteric artery
T4 = Tumour involves the coeliac axis or the superior mesenteric artery (unresectable primary tumour)
Regional lymph nodes (N)
NX = Regional lymph nodes cannot be assessed
N0 = No regional lymph node metastasis
N1 = Regional lymph node metastasis
Distant metastasis (M)
M1 Distant metastasis

ESTADIFICACIÓN DEL CÁNCER DE PÁNCREAS (AJCC)			
Estadio	T	N	M
Estadio 0	Tis	N0	M0
Estadio IA	T1	N0	M0
Estadio IB	T2	N0	M0
Estadio IIA	T3	N0	M0
Estadio IIB	T1,T2,T3	N1	M0
Estadio III	T4	Cualquier N	M0
Estadio IV	Cualquier T	Cualquier N	M1

Fuente: AJCC Cancer Staging Handbook, Séptima edición (2010) publicado por Springer Science and Business Media LLC.(www.springer.com)

1.5 Tratamiento del cáncer de páncreas

Hasta la fecha, la única modalidad terapéutica potencialmente curativa en el CaPa es la cirugía. Pero, la mayoría de los casos son irresecables, y tan sólo el 20% de los cánceres de páncreas son candidatos a la cirugía(17). Para los tumores de cabeza de páncreas la técnica quirúrgica de elección es la duodenopancreatectomía cefálica de Whipple; mientras que para los tumores de cuerpo y cola la técnica de elección es la

pancreatectomía distal o total con o sin esplenectomía. El CaPa se puede clasificar según la Asociación Americana Hepato-Pancreato-Biliar: como resecable, resecable en el límite, localmente avanzado o enfermedad metástica. Se debe tomar una decisión de tratamiento de acuerdo con estos hallazgos, incluidos los aspectos generales y nutricionales del paciente (Figura 3)(3,6).

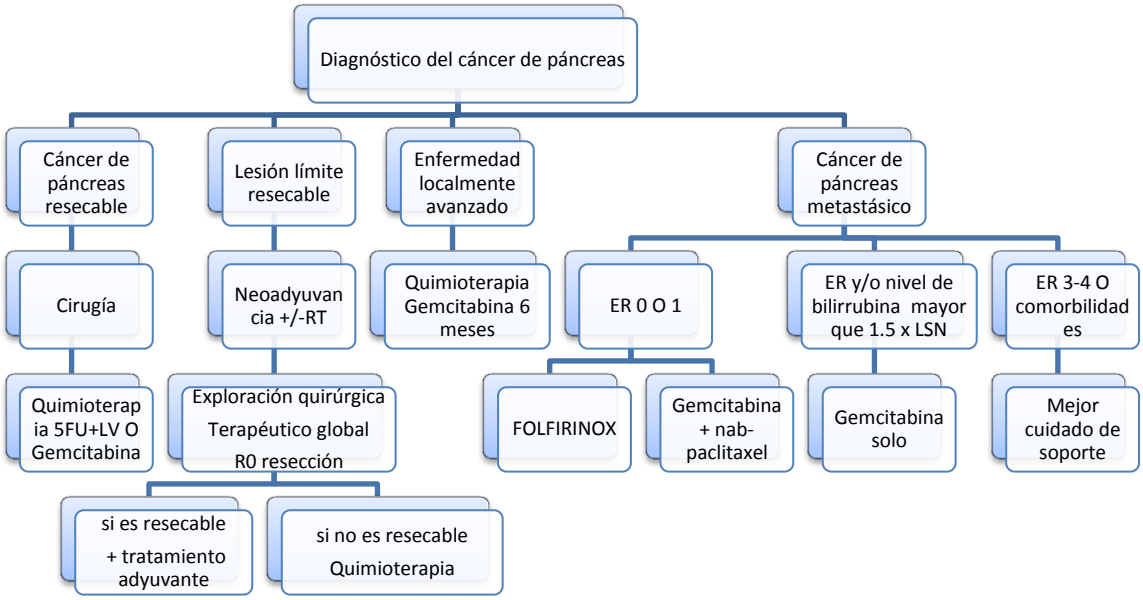

Figura 3. Estrategia de tratamiento del cáncer de páncreas. (Fuente: Adaptado de ESMO Clinical Practice Guidelines). RT: radioterapia, ER: estado de rendimiento. LV: leucovorina, 5-FU: 5-fluorouracil, LSN:límite superior de lo normal.

1.5.1 Tratamiento de la enfermedad localizada.

Las guías internacionales de consenso para el tratamiento oncológico del CaPa localizado recomiendan lo siguiente:

-Es necesario un abordaje multidisciplinar.

-Se debe alcanzar la resección del tumor para los siete márgenes identificados por el cirujano.

-La linfadenectomía estándar debe implicar la extirpación de ≥15 ganglios linfáticos para permitir una estadificación patológica adecuada de la enfermedad(17).

-El tratamiento adyuvante se realiza con 6 ciclos de gemcitabina o ácido folínico combinado con 5-FU(18).

-No se debe administrar quimiorradiación a los pacientes después de la cirugía, excepto en ensayos clínicos(18).

1.5.2 Tratamiento de la enfermedad no resecable: lesiones límite resecables

Las guías clínicas recomiendan que los pacientes con lesiones límite resecables deben incluirse en ensayos clínicos siempre que sea posible. En la práctica habitual, si el paciente no está incluido en un ensayo, un período de quimioterapia neoadyuvante basado en gemcitabina o FOLFIRINOX (ácido folínico, 5-fluorouracilo, irinotecán y oxaliplatino) seguido de quimiorradiación y posteriormente la cirugía parece ser la mejor opción(19).

1.5.3 Tratamiento de la enfermedad no resecable: enfermedad localmente avanzada

En este sentido las guías internacionales proponen que:

-La quimioterapia estándar basada en gemcitabina debe ser de 6 meses.

-Se ha observado un papel menor de la quimiorradiación en este subgrupo de pacientes.

-Es imposible recomendar cualquier tratamiento de quimiorradiación que no sea la combinación clásica de capecitabina y radioterapia.

1.5.4 Tratamiento de la enfermedad metastásica

En este caso en el que el tumor es irresecable, la supervivencia es bastante limitada así como las opciones de tratamiento efectivas:

-Cuidados paliativos y de apoyo: la obstrucción duodenal es preferiblemente manejado por la colocación endoscópica de un *stent* de metal expansible cuando sea posible(20).

-Para pacientes con un estado funcional (ECOG, *Eastern Cooperative Oncology Group*) de 3/4, con una significativa morbilidad y una esperanza de vida muy corta sólo el tratamiento sintomático debe ser considerado.

-En pacientes muy seleccionados con el estado funcional ECOG=2 y una gran carga tumoral, se puede considerar la gemcitabina y el nab-paclitaxel (nano-albumin bound paclitaxel) para un mayor grado de respuesta.

-Para pacientes con un estado funcional ECOG=2 y/o un nivel de bilirrubina 1.5 veces superior a los niveles normales (LSN), se podría considerar una monoterapia con gemcitabina.

-Si el estado funcional del paciente ECOG= 0 o 1 y el nivel de bilirrubina está por debajo de 1,5 veces el límite superior de lo normal (LSN), se deben considerar dos tipos de quimioterapia combinada: el régimen de FOLFIRINOX o la combinación de gemcitabina y nab-paclitaxel(21).

1.5.5 Inmunoterapias y terapias dirigidas

Actualmente hay datos limitados disponibles para apoyar el uso de inmunoterapia para el CaPa. Lamentablemente, el ensayo clínico con el inhibidor del punto de control inmunitario (anti-PD-L1) no demostró eficacia de la terapia en pacientes con CaPa avanzado, lo que se ha atribuido a la baja inmunogenicidad y al microambiente tumoral inmunosupresor de este tipo de cáncer(22). Sin embargo, el análisis del perfil genómico integral ha encontrado deficiencias en pequeños subconjuntos de pacientes que pueden ser objeto de intervención, en particular aquellos con mutación en *BRCA1/2* y/o en el sistema de reparación de errores genéticos (MMR, el inglés *Mismatch Repair*). Por ejemplo, se ha descrito que los tumores deficientes en MMR eran más susceptibles a la inmunoterapia en múltiples tumores sólidos, incluido el CaPa, lo que llevó a la aprobación de la FDA de pembrolizumab (anti-PD-1) para pacientes con enfermedad avanzada que tienen esta mutación(23). De hecho, las guías clínicas para el tratamiento y manejo del CaPa avanzado ahora recomiendan realizar pruebas para detectar mutaciones en MMR a pesar de su baja prevalencia, debido al potencial de remisión de la enfermedad, y recomiendan el pembrolizumab como tratamiento de segunda línea en pacientes positivos para la mutación en genes de MMR(23). Mientras tanto, el ensayo clínico de fase III POLO, mostró la eficacia de olaparib, un inhibidor de PARP, como terapia de mantenimiento en pacientes que tenían una mutación en la línea germinal de *BRCA1/2* (supervivencia libre de progresión: 7,4 meses con olaparib, frente a 3,8 meses con placebo, P=0,004)(24). En consecuencia, olaparib se encuentra actualmente bajo

revisión de la FDA como terapia de mantenimiento en este subconjunto de pacientes. El perfil genómico completo tiene el potencial de permitir la identificación de pacientes con alteraciones específicas que pueden ser candidatos para inmunoterapia y terapias dirigidas en el futuro. Finalmente, las terapias combinadas que apuntan a reprogramar el microambiente inmunosupresor del tumor junto con la inmunoterapia también se están investigando y han arrojado algunos resultados preliminares alentadores(25).

2. Las proteínas de unión a RNA

Las proteínas de unión a ARN (RBPs, del inglés *RNA Binding Proteins*) son abundantes y se expresan de forma ubicua en las células. Desempeñan un papel central y conservado en la regulación de genes(26), y actúan como importantes participantes y coordinadores para mantener la integridad del genoma(27). Los RBPs tienen amplias capacidades cuyo resumen se encuentra en la Figura 4(27,29).

Hay gran cantidad de RBPs humanas, pero muy pocas se han estudiado en profundidad, como AGO2, Nova, PTB, HuR, AUF1, TTP o CUGBP2, que son conocidas por su papel en muchos procesos de regulación, incluida la interacción con el ARN no codificante(29), el control de la localización intracelular de ARN no codificantes(30), la metilación(31) o formando el complejo de silenciamiento inducido de ARN (RISC, del inglés *RNA induced silencing complex*)(32). Las RBPs participan en procesos biológicos integrales, como el desarrollo reproductivo, la tumorigénesis y la apoptosis, y por lo tanto están estrechamente relacionadas con cáncer. Un estudio funcional sistemático de las RBPs será útil para comprender la función y el mecanismo del ARN no codificante, pero también tendrá un valor aplicado significativo en el estudio de la patogénesis del cáncer y en la detección de nuevas dianas terapéuticas.

Por lo tanto, la presente Tesis Doctoral se centra en dos tipos de proteínas de unión a RNA: PIWI y UNR, para estudiar las posibles vías asociadas con el desarrollo y progresión del CaPa e identificar posibles mecanismos moleculares, marcadores de diagnóstico, pronóstico, y posibles dianas terapéuticas.

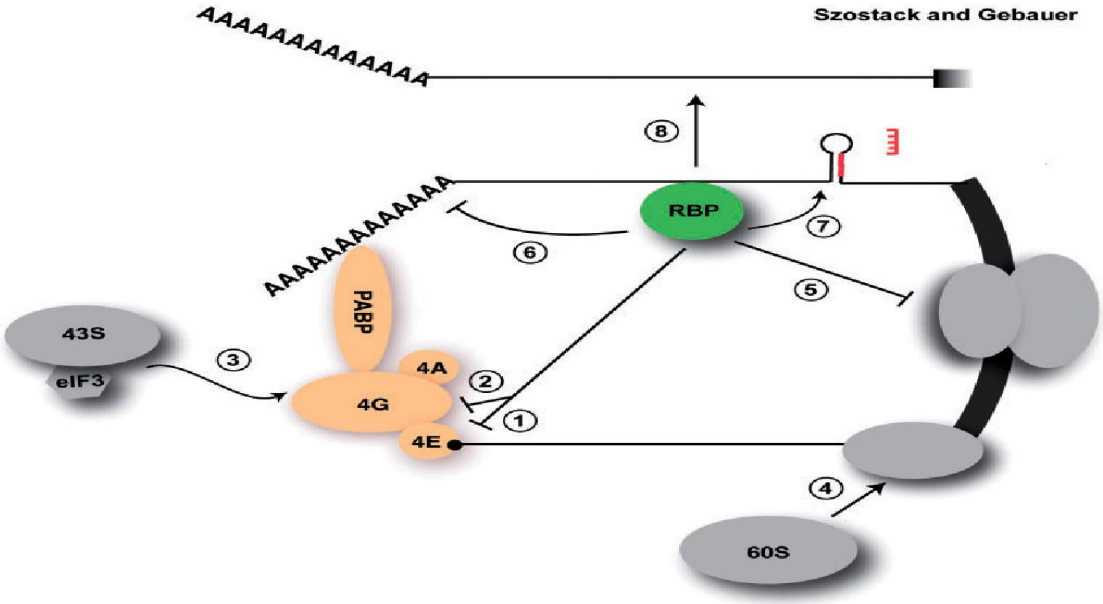

Figura 4. Mecanismos de regulación traduccional por RBPs. 1) las RBP se unen a otros factores que compiten por eIF4E dificultando la formación del complejo; 2) RBP inhibe la traducción debido a su baja afinidad por eIF4G; 3) RBP interfiere con la interacción entre eIF4G y eIF3, que conduce a la inhibición del reclutamiento del ribosoma; 4) también inhibe la unión de la subunidad ribosómica 60S al complejo 43S colocado; 5) la mayoría de las RBP regulan la iniciación de la traducción; 6) las RBP son capaces de unirse a la poly(A) y llevar a cabo una desadenilación; 7) también regulan el procesamiento de ANR pequeños no codificantes; 8) e interaccionan con mRNA facilitando o retardando su traducción(28).

2.1 Proteínas PIWI

Las proteínas PIWI (del inglés, *P-element-induced wimpy testis*) pertenecen a la familia Argonauta (AGO) y se expresan principalmente en las células de la línea germinal(33). Las proteínas AGO juegan un papel importante en la regulación de la expresión génica a través del reconocimiento complementario de pequeños fragmentos de ARN no codificantes, que los guían contra sus genes diana(34). Recientemente, las proteínas PIWI han participado en la remodelación epigenética y en la meiosis de la línea germinal(35). Específicamente, las proteínas PIWI reconocen y se unen a un tipo de ARN pequeños no codificantes llamados piRNA (del inglés: *PIWI associated RNA*), que constituye el Complejo Silenciador Inducido por piRNA (piRISC). Estas proteínas tienen papeles importantes en la regulación epigenética, el silenciamiento de elementos transponibles, la protección de la integridad del genoma, la gametogénesis y la biogénesis de los propios piRNA(36).

La familia de proteínas PIWI está altamente conservada en una variedad de organismos(37). De hecho, hay cuatro proteínas PIWI humanas: PIWIL1 (también conocida como HIWI), PIWIL2 (HILI), PIWIL4 (HIWI2) y PIWIL3 (HIWI3) (Tabla 2)(38).

Tabla 2. Ubicación cromosómica y masa molecular de las proteínas PIWI.

Proteína	Locus genómico	Masa molecular (kDa)
PIWIL1	12q24.33	98.5
PIWIL2	8p21.3	110
PIWIL3	22q11.23	101
PIWIL4	11q21	97

PIWI: P element induced wimpy testis; PIWIL: PIWI like. kDa: kiloDalton(39).

En los últimos años, las proteínas PIWI se han relacionado con algunas de las características distintivas del cáncer, como la proliferación celular, el mantenimiento de la integridad genómica, la evasión de la apoptosis, la invasión y la metástasis(40,41). Esto sugiere que podrían usarse para el diagnóstico, pronóstico y quizás el tratamiento del cáncer. Por ello, el número de estudios que muestran diferentes patrones de expresión en muestras sanas y tumorales de distintos tipos de tumores está aumentando.

Los piRNA son los miembros más nuevos de la familia de los ARN no codificantes (ncRNA). En el genoma humano se encuentran alrededor de 23.000 piRNA, además el número de piRNA es mucho más elevado que el de microRNA (~2.000)(42). Esto indica que los piRNA pueden estar involucrados en la regulación génica, pero su mecanismo específico está por estudiar en profundidad. Los piRNA tienen una estructura única de 2'-O-metilo en el extremo 3' UTR, y se ha demostrado que esta propiedad es específica de ellos(37).

Es importante conocer la biogénesis de los piRNA, sus funciones, sus mecanismos moleculares subyacentes y su papel emergente en la carcinogénesis.

2.1.1 Biogénesis de piRNA

Los piRNA maduros tienen una longitud de 26-30 nucleótidos y están cerca de la longitud de los microRNA (20-24 nucleótidos) y de los siRNA (21-25 nucleótidos). Los precursores de los piRNAs se transcriben grandes (hasta 200 kilobases); a partir de los precursores monocatenarios independientemente de la endoribonucleasa DICER. Por el

contrario, los microRNAs y siRNAs son procesados por DICER a partir de precursores bicatenarios(43). Estos precursores generalmente se generan de ciertos *loci* genómicos específicos que contienen elementos repetitivos como ocurre con los transposones(44). Además, los precursores de los piRNA requieren modificación postranscripcional para convertirse en piRNA maduros. La biogénesis de los piRNA implica dos vías principales: la vía de amplificación primaria y una vía de amplificación secundaria, también conocida como el Ciclo de Ping-Pong (Figura 5)(45).

2.1.1.1 Amplificación primaria

Los piRNA se derivan de un número relativamente pequeño de regiones genómicas, denominadas agrupaciones de piRNA. Curiosamente, la mayoría de estos grupos consisten en varios elementos de ADN transponibles, lo que indica que los piRNA son potencialmente antisentido de ciertos retrotransposones, y proporcionan pistas sobre cómo éstos podrían afectar la función celular(30,46). La síntesis primaria se basa en la ARN polimerasa II del núcleo que transcribe pequeñas secuencias de nucleótidos para formar un largo precursor piRNA monocatenario, que luego se transfiere al citoplasma. El fragmento precursor de piRNA producido, después, es procesado hasta obtener su longitud final mediante exo-escisión de 3' a 5', para luego unirse por separado a la proteína PIWI para formar un complejo de proteína piRNA/PIWI(47).

Después de que se forme el complejo de proteína piRNA/PIWI, éste migra de regreso al núcleo para alcanzar el gen objetivo, y por complementariedad de bases entre el piRNA y el ADN, activa su mecanismo de silenciamiento y bloquea la transcripción del gen objetivo. Los piRNA además de ser reguladores transcripcionales que actúan sobre elementos transponibles, también actúan mediante el reclutamiento de metiltransferasas de histonas, lo que resulta en la modificación de la heterocromatina para el silenciamiento transcripcional(48).

2.1.1.2 Amplificación secundaria (Ciclo de Ping-Pong)

Después de la generación de piRNA primarios, la amplificación secundaria se inicia en el citoplasma a través de lo que se denomina un mecanismo de "Ping-Pong". Brevemente, el complejo de proteína piRNA/PIWI reconoce sus ARN mensajeros diana por complementariedad y utiliza la actividad de nucleasa de PIWI para recortar los extremos 3´ de los piRNA primarios, lo que conduce a la producción de piRNA antisentido

secundarios. Como sugiere el nombre de Ping-Pong, los piRNA antisentido se combinan con proteínas PIWI y una vez más, se dirigen a los precursores de piRNA complementarios para producir piRNA con sentido. A través de este ciclo que depende de secuencias complementarias, los piRNA se amplifican y acumulan en el citoplasma (Figura 5)(36,47).

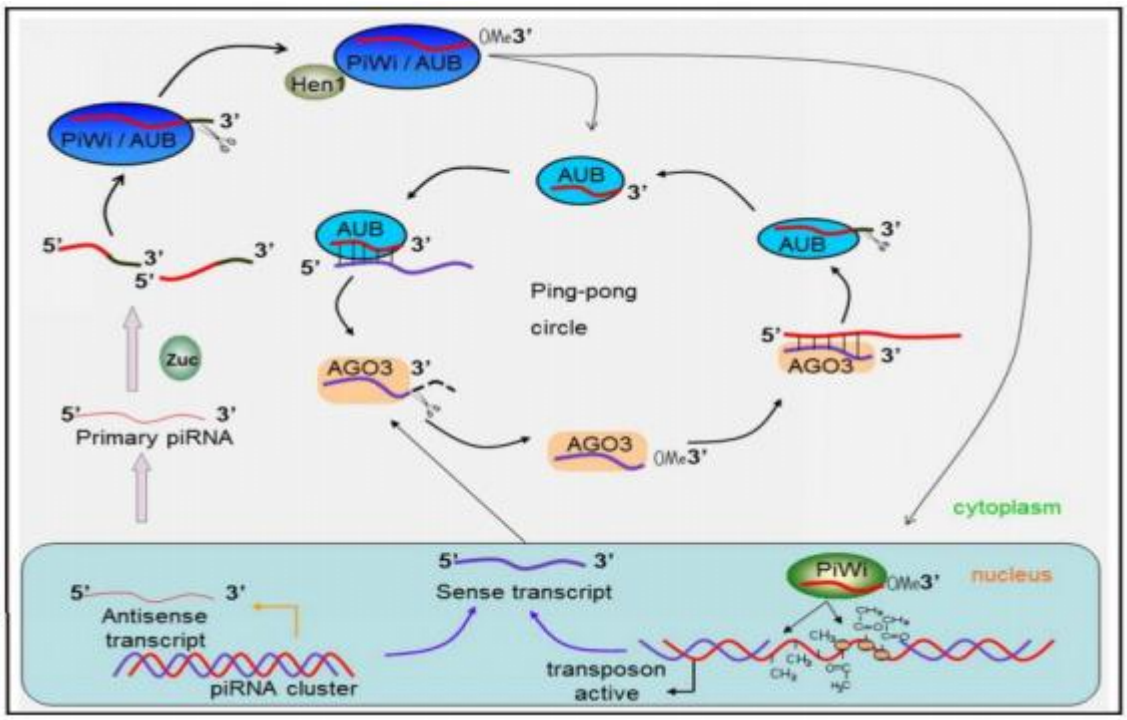

Figura 5. Biogénesis de los piRNA(41). Zuc: riboendonuclease Zucchini, Hen1: Small RNA 2'-O-methyltransferase, AUB: Protein aubergine, AGO3: Protein argonaute-3, PIWI: P element induced wimpy testis.

2.1.2 Las proteínas PIWI en cáncer

Las proteínas PIWI se descubrieron por primera vez en *Drosophila sp.*, donde se observó que estaban involucradas en el mantenimiento y la autorrenovación de las células madre de la línea germinal(49). PIWI es una proteína que participa en el silenciamiento de los retrotransposones y el control de la movilidad de la línea germinal masculina. Además, las PIWI también está involucradas en la producción de esperma(50). Se ha demostrado que las mutaciones knock-out de las proteínas PIWI pueden provocar defectos en el desarrollo de esperma(51). Por lo tanto, estas proteínas son factores implicados estrechamente en la regulación de la línea germinal y las células madre.

Algunos estudios demuestran que existe una fuerte correlación entre la expresión de las proteínas PIWI y el mal pronóstico clínico, por lo que la investigación actual se centra en estudiar sus mecanismos de tumorigénesis. Varios estudios han demostrado que la alta expresión de las proteínas PIWI se asocia a carcinoma de células escamosas esofágicas, al cáncer gástrico, cáncer de hígado, colangiocarcinoma, cáncer intestinal, cáncer de mama, cáncer de pulmón, carcinoma de células renales, cáncer de vejiga, cáncer de ovario y a melanoma. También se ha demostrado que las proteínas PIWI están involucradas en la proliferación de células cancerosas, en la regulación de la apoptosis, la invasión y metástasis; y pueden actúar como potenciales biomarcadores de diagnóstico y pronóstico en varios tipos de cáncer (Tabla 3).

2.1.2.1 PIWIL1 (HIWI)

PIWIL1 (*Piwi like RNA-mediated gene silencing 1*) puede desempeñar un papel crucial en la vía de señalización del cáncer gástrico y puede ser útil como objetivo terapéutico de este cáncer(52). PIWIL1 se localiza principalmente en el citoplasma de las células tumorales. La alta expresión de PIWIL1 en el tejido tumoral de cáncer colorrectal está estrechamente relacionada con el grado de diferenciación tumoral, la profundidad de infiltración, la invasión vascular, la metástasis en los ganglios linfáticos y un mayor estadio TNM. Además, PIWIL1 puede inducir el mecanismo de transición epitelio-mesenquima (TEM) en células de cáncer de endometrio, aumentar su viabilidad celular, la migración, la invasión y la actividad formadora de esferas con fenotipo de célula madre de cáncer (CSC). También, la sobreexpresión de PIWIL1 conduce a una mayor expresión de ciertos marcadores de CSC endometriales conocidos como son: CD44 y ALDH. Por lo tanto, PIWIL1 puede convertirse en un objetivo valioso para desarrollar una nueva estrategia de tratamiento para la cáncer de endometrio(53).

PIWIL1 está regulado por la hipometilación de ADN, se sobreexpresa en los tejidos tumorales pulmonares, lo que podría facilitar la proliferación, invasión y migración de las células cancerosas y contribuir a una peor supervivencia global en pacientes con adenocarcinoma de pulmón. Por ello, cabe destacar que PIWIL1 puede ser un objetivo potencial para el tratamiento del cáncer de pulmón como regulador epigenético(54). Además, la expresión de PIWIL1 es significativamente mayor en el carcinoma ductal invasivo, que promueve el desarrollo del cáncer mediante la metilación

aberrante del ADN, lo que resulta en el silenciamiento genómico e induce un estado de CSC(55). En CaPa, la expresión de PIWIL1 se evaluó previamente en 56 muestras a niveles de ARNm y proteína. Este estudio no mostró impacto en la supervivencia de los pacientes ni por los niveles elevados de la proteína PIWIL1, ni por los niveles de expresión de ARNm. Sin embargo, la expresión de ARNm alterada, es decir, tanto la expresión baja como la alta en comparación con la expresión intermedia, presentó un mal pronóstico sólo en la cohorte de pacientes hombres (P=0,034)(55).

2.1.2.2 PIWIL2 (HILI)

PIWIL2 (*Piwi like RNA-mediated gene silencing 2*) está sobreexpresado tanto a nivel de ARNm como de proteína en tejidos tumorales malignos de carcinoma de pulmón en comparación con tejido normal adyacente. Además, se ha demostrado que promueve la proliferación celular al aumentar la expresión de CDK2 y Ciclina A, que son factores esenciales que controlan la síntesis de ADN y el ciclo celular. El silenciamiento de PIWIL2 desencadena apoptosis y la detención del ciclo celular en la fase G2/M(56). Asimismo, la baja expresión de PIWIL2 está relacionada con una corta supervivencia en pacientes con cáncer colorrectal(57). PIWIL2 está altamente expresado en células de glioma y su expresión se correlaciona positivamente con un mal pronóstico del paciente. La pérdida de expresión de PIWIL2 en las células de glioma induce la parada del ciclo celular, aumenta la apoptosis e inhibe la migración de células(58).

2.1.2.3 PIWIL3 (HIWI3)

PIWIL3 (*Piwi like RNA-mediated gene silencing 3*) se expresa en cáncer de ovario estadio III, y su expresión en el tumor primario es mayor en comparación con sus tejidos normales adyacentes (P<0.01), y dicha expresión es todavía mayor en los focos metastásicos(59). PIWIL3 también se considera un biomarcador pronóstico en cáncer de mama, ya que su regulación positiva se asoció significativamente con la supervivencia libre de progresión (P=0.01) y la supervivencia global (P=0.02)(60). Además, PIWIL3 parece jugar un papel crucial en la progresión de melanoma y su expresión es mayor cuanto mayor es el estadio tumoral(61). En los cánceres gastrointestinales, la expresión de PIWIL3 también es mayor en los tejidos tumorales en comparación con sus tejidos sanos(62). Por el contrario, PIWIL3 parece tener un efecto protector en glioma debido a

que la sobreexpresión reduce la proliferación, migración e invasión de células tumorales *in vitro* y disminuye el tamaño del tumor en modelos *in vivo*(63).

2.1.2.4 PIWIL4(HIWI2)

El papel de PIWIL4 (*Piwi like RNA-mediated gene silencing 4*) está asociado a modificaciones de la cromatina en las células somáticas humanas(64), y es capaz de procesar los precursores para generar varios ARN pequeños no codificantes en ausencia de DICER(65). La falta de expresión de PIWIL4 podría estar relacionada con el desarrollo de diabetes tipo 2, ya que su regulación negativa en las células beta-pancreáticas dio como resultado una secreción defectuosa de insulina(66). Sin embargo, su función en la tumorigénesis es bastante controvertida. Por un lado, se encuentra una alta expresión de PIWIL4 en los tejidos tumorales de cáncer colorrectal(67), cáncer cervical(68), cáncer gástrico(69) y en focos primarios y metastásicos de cáncer de ovario(59), en comparación con sus tejidos sanos adyacentes. Su desregulación no sólo mejoró significativamente el efecto apoptótico de los tratamientos en el tumor de células de Leydig(70), sino también aumentó la apoptosis, la migración y la invasión de células de cáncer de mama *in vitro*(70). En el carcinoma hepatocelular, la expresión nuclear de PIWIL4 junto con la expresión de PIWIL2 se ha asociado a un peor pronóstico(71).

Por el contrario, otros estudios han informado que la baja expresión de PIWIL4 se asoció significativamente con un peor pronóstico en el carcinoma hepatocelular(72), en sarcoma de partes blandas(73), en cáncer de pulmón de células no pequeñas(74) y en carcinoma de células renales(57). También se encontraron niveles bajos de PIWIL4 en tejidos de carcinoma hepatocelular(72) y en otros tumores como el de mama(60) y cáncer de pulmón de células no pequeñas(74) en comparación con los tejidos sanos colindantes. Además, se ha descrito en tumores testiculares que la falta de expresión de PIWIL4 está causada por la hipermetilación de la isla CpG de su promotor(75).

Tabla 3. El papel de las proteínas PIWI en varios tipos de cáncer.

PIWI	Cáncer	Expresión	Función	PMID
PIWIL1	Cáncer de pulmón	up	Hipometilación del ADN	29168346
	Cáncer gástrico	up	Regula la vía de señalización del cáncer gástrico.	30598579
	Cáncer colorrectal	up	Es utilizado como un marcador molecular importante para predecir el pronóstico de pacientes con CCR.	28634417
	Cáncer de células renales	down	Sirve como biomarcador pronóstico potencial en pacientes con CCR	26811690
	Cáncer endometrial		Puede convertirse en un objetivo para desarrollar un tratamiento novedoso;	26506848
		up	Metilación del ADN.	26056945
	Carcinoma ductal invasivo		Metilación aberrante de ADN.	29599319
	Cáncer de mama	up	Función proteica con papel oncogénico en el cáncer de mama.	25292027
	Glioma	up	La reducción de PIWIL1 inhibió el crecimiento tumoral *in vivo*, PIWIL1 actuó	25269862
		up	como un oncogén para participar en la progresión del glioma.	
			El PIWIL1 puede ser un factor clave en la progresión del glioma y podría usarse como un marcador molecular potencial para los gliomas malignos en el diagnóstico patológico y la evaluación del pronóstico.	21138738
	Carcinoma hepatocelular		PIWIL1 puede desempeñar un papel esencial en la progresión del carcinoma	25370791
		up	hepatocelular y puede ser el objetivo de la terapia contra el cáncer.	
			PIWIL1 puede desempeñar un papel clave en la proliferación y metástasis de CHC, por lo que podría ser un factor pronóstico potencial para CHC, especialmente en subtipos bien diferenciados.	21989785
	Seminoma		La proteína PIWIL1 se relacionó con el seminoma debido al papel esencial	12037681
		expresión	que juega PIWIL1 en la proliferación de células germinales.	
	Cáncer de ovarios	ectópica down	La sobreexpresión de PIWIL1 reduce la invasividad de la línea celular de cáncer de ovario SKOV3.	24932571
PIWIL2	Glioma	up	Correlacionado con el mal pronóstico.	28534979
	Cáncer de cuello	up	Indujo acetilación de H3K9 pero redujo la trimetilación de H3K9.	27602489
	uterino Cáncer de mama	up	La combinación de piR-932 y PIWIL2 podría ser el objetivo potencial para bloquear la metástasis del cáncer de mama a través de la promoción de la metilación de Latexin.	23992744

	Cáncer de pulmón de células no pequeñas	up	Aumenta la expresión de CDK2 y Ciclina A.	26373553
	Cáncer de células renales	down	Se asocia con peor supervivencia.	26811690
	Cáncer colonrectal	up	Las células positivas para PIWIL2 juegan un papel positivo en la progresión del cáncer colorrectal.	23110023
	Carcinoma hepatocelular	nuclear co-expression	PIWIL2 tenía el potencial de ser un marcador molecular para el juicio pronóstico del CHC.	27894076
PIWIL3	Glioma	down	Regula la vía PIWIL3/piR-30188/OIP5-AS1/miR-367-3p/CEBPA/ TRAF4. Regula la ruta de señalización JAK2/STAT3.	29464001
	Cáncer gástrico	up	Participa en la progresión MM y metastásico.	28868440
	Mieloma múltiple	up	Se ha identificado que la proteína PIWIL3 en la ruta de piRNA desregulada	27858163
	Cáncer de mama	up	actúa como un marcador para el pronóstico del cáncer de mama.	27177224
PIWIL4	Cáncer de mama triple negativo	up	Activa la señalización de TGF-β/MAPK/ERK y FGF, evitando el reconocimiento immune.	26957540
	Cáncer de mama	down	Se ha identificado que la proteína PIWIL4 actúa como un marcador para el pronóstico del cáncer de mama.	27177224
	Carcinoma hepatocelular	nuclear co-expression	PIWIL4 tiene el potencial de ser un biomarcador pronóstico del CHC.	27894076
	Cáncer Cervical	up	PIWIL4 puede jugar un papel cancerígeno en el cáncer cervical a través de la vía P14ARF/P53 y puede servir como un nueva diana terapéutica para el futuro.	22483988

CCR: cáncer colorrectal, CHC: Carcinoma hepatocelular, CDK2:Cyclin-dependent kinase 2, OIP5-AS1:OIP5 ARN antisentido 1, JAK2: Janus kinase 2, TGF-β: Factor de crecimiento transformante beta, MAPK: Proteína quinasa activada por mitógeno ERK : Quinasas reguladas por señal extracelular,FGF: Factor de crecimiento de fibroblastos.

2.2 La proteína UNR

2.2.1 Función de la proteína UNR

La proteína UNR (*Upstream-of-N-Ras*), codificada por el gen *CSDE1* (cold-shock domain containing E1) en mamíferos, es una RBP conservada con dominios que permiten la unión a ADN y ARN monocatenario(76). UNR tiene una amplia expresión en tejidos y tipos de células tanto normales como tumorales(77,78). UNR se localiza principalmente en el citoplasma y ciertos estudios recientes revelaron que UNR está involucrada en la regulación de la traducción de varios ARNm(79,80,81). También, UNR se ha caracterizado como un regulador en los mecanismos de compensación de la dosis del cromosoma X(82). UNR también es un factor que se requiere para el inicio interno de la traducción (83) mediado por IRES (*internal ribonsome entry sites*)(84,85). UNR estimula la traducción dependiente de IRES de c-myc, la quinasa PISTLRE del ciclo celularny Apaf-1(86).

2.2.2 La proteína UNR en cáncer

En cáncer, se ha demostrado que UNR regula protooncogenes como c-fos(80) y c-myc(87). Además, UNR promueve la progresión del melanoma al regular la expresión de PTEN, RAC1 o VIMENTIN entre otros(88). En el cáncer colorrectal, la desregulación de UNR reduce la viabilidad celular y la migración a través de una restricción de la transición epitelio-mesenquimal y aumenta la sensibilidad a la apoptosis; mientras que la alta expresión de UNR se asoció con un mal pronóstico y se correlacionó positivamente con la expresión de c-MYC(89). La eliminación de la expresión de HEPSIN, un protooncogen sobreexpresado en cáncer de próstata, condujo a un aumento de UNR y la regulación positiva de su actividad IRES(90).Por otra parte, la expresión de UNR desregula la expresión de NRAS a través de la acumulación de su ARNm en los tejidos, pero lo sobreepresa en células de linfomas B debido a su capacidad para regular inserciones provirales(91,92). No se han obtenido ratones transgénicos homocigóticos negativos para la expresión UNR debido al efecto letal embrionario que conlleva su eliminación(91). En conjunto, estos datos apuntan a diversos papeles cruciales de UNR en el desarrollo del cáncer.

OBJETIVOS

Nuestra hipótesis es que las proteínas de unión a RNA, PIWI y UNR son potenciales biomarcadores de pronóstico en CaPa y pueden desempeñar funciones cruciales en el origen y desarrollo de CaPa.

Para confirmar o rechazar esta hipótesis se plantearon los siguientes objetivos para la presente Tesis Doctoral:

1. Evaluar la expresión de las 4 proteínas PIWI en líneas celulares de CaPa.

2. Conseguir un alto silencimiento de aquellas proteínas PIWI sobreexpresadas.

3. Estudiar los posibles mecanismos o factores asociados a estas proteínas mediante experimentos funcionales.

4. Evaluar la expresión de las cuatro proteínas PIWI en muestras de pacientes con CaPa y asociar su expresión a la supervivencia global y libre de progresión.

5. Evaluar la expresión de la proteína UNR, en muestras de pacientes de CaPa y asociar su expresión al pronóstico de los pacientes.

6. Buscar factores asociados a UNR que puedan explicar un posible mecanismo molecular.

RESULTADOS

Publicaciones Científicas

ARTÍCULO 1: The Prognosis Value of PIWIL1 and PIWIL2 Expression in Pancreatic Cancer

El CaPa es una manifestación altamente agresiva del cáncer, y actualmente presenta un resultado clínico pobre debido a su diagnóstico tardío con enfermedad metastásica. La cirugía es el único enfoque con una intención curativa, pero después de la cirugía, faltan biomarcadores de pronóstico y predictivo de respuesta al tratamiento.

En este trabajo, nuestro objetivo fue evaluar una asociación entre la expresión de PIWIL1 y PIWIL2 y el pronóstico de los pacientes con cáncer biliopancreático. Para ello, analizamos la expresión de proteínas en muestras de tumor resecado completo, y encontramos una asociación significativa entre la expresión de PIWIL2 y la supervivencia libre de progresión y global (P=0.036 y P=0.012, respectivamente). Sin embargo, la expresión de PIWIL2 se asoció significativamente con la supervivencia libre de progresión (P=0.029), y la supervivencia general (P=0.025) de tales tumores se originó en el páncreas, pero no en el conducto biliar o la ampolla de Vater. Un análisis posterior reveló que PIWIL1 y PIWIL2, tanto en los niveles de expresión de ARNm como de proteína, se correlacionaron positivamente con factores asociados al subtipo molecular progenitor de cáncer pancreático.

En base a estos hallazgos, la expresión de PIWIL1 y PIWIL2 puede considerarse un biomarcador pronóstico potencial para el CaPa resecable y puede servir para guiar las decisiones de tratamiento adyuvante posteriores.

Aportación Personal al trabajo:

En este trabajo mi aportación se centró en llevar a cabo la selección de pacientes con cáncer biliopancreático así como la búsqueda de sus tejidos parafinados y se construyeron microarrays de tejidos (TMA). Además, realicé los experimentos de inmunohistoquímica. También me encargué de la parte de análisis estadístico. Finalmente, contribuí a la redacción del artículo y de la posterior revisión del mismo tras las correcciones oportunas de mis directores de tesis.

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The Prognosis Value of PIWIL1 and PIWIL2 Expression in Pancreatic Cancer

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Abstract: Pancreatic cancer is a highly aggressive manifestation of cancer, and currently presents poor clinical outcome due to its late diagnosis with metastasic disease. Surgery is the only approach with a curative intend; however, the survival rates seen in this type of patient are still low. After surgery, there is a lack of predictive prognosis biomarkers to predict treatment response and survival to establish a personalized medicine. Human P-element-induced wimpy testis 1 (PIWIL1) and P-element-induced wimpy testis 2 (PIWIL2) proteins act as protectors of germline, and their aberrant expression has been described in several types of tumors. In this study, we aimed to assess an association between PIWIL1 and PIWIL2 expression and the prognosis of biliopancreatic cancer patients. For this, we analyzed protein expression in complete resected tumor samples, and found a significant association between PIWIL2 expression and both progression-free and overall survival ($p = 0.036$ and $p = 0.012$, respectively). However, PIWIL2 expression was significantly associated with progression-free survival ($p = 0.029$), and overall survival ($p = 0.025$) of such tumors originated in the pancreas, but not in the bile duct or ampulla of Vater. Further analysis revealed that PIWIL1 and PIWIL2, at both mRNA and protein expression levels, correlated positively with factors associated to the progenitor molecular subtype of pancreatic cancer. Based on these findings, PIWIL1 and PIWIL2 expression may be considered a potential prognostic biomarker for resectable pancreatic cancer and may serve to guide subsequent adjuvant treatment decisions.

Keywords: PIWI proteins; PIWIL1; PIWIL2; pancreatic cancer; prognostic biomarker; molecular subtypes

1. Introduction

Pancreatic cancer (PC) is one of the tumors with higher incidence in developed countries [1]. It is the fourth leading cause of cancer death in both sexes in the USA, and incidence continues to increase. Around 56,770 new cases of PC are estimated in the USA in 2019 (29,940 cases in men and 26,830 in women), and 45,750 deaths are estimated in the USA in 2019 (23,800 in men and 21,950 in women) [2]. PC is the eighth leading cause of cancer deaths in men and the ninth in women worldwide [3]. Indeed, the incidence of PC is expected to surpass breast, prostate, and colorectal cancers to become the second

cause of cancer-related death by 2030 [4]. Although the five-year survival rate is 50% when tumors are <2 cm in size and close to 100% for tumors <1 cm [5], PC is normally asymptomatic, and it is often diagnosed at metastasic stages [6]. This fact drastically reduces patient survival to 3% of patients [2,7]. Ampullary adenocarcinoma is considered the tumor with the best prognosis of the biliopancreatic region. It is a relatively uncommon tumor and represents 0.2% of all digestive tumors [8]. The long-term prognosis is variable with survivals ranging between 37–75% at five years [9]. Regarding bile duct carcinomas, they present very low incidence (1–2 per 100,000 population) [10], and differentiation from ampullary adenocarcinoma is not easy to perform. Median survival of tumors originated in the bile duct is around 29 months, and the five-year survival rate is 27% [11]. To date, surgical resection with pancreatoduodenectomy (Whipple procedure) is considered the best procedure to manage tumors originated in the ampulla of the Vater, bile duct, or head of the pancreas. When tumors are localized in the tail or body of the pancreas, a distal pancreatectomy is performed and some cases required total pancreatectomy. Adjuvant treatment for complete resected patients (R0) is based on gemcitabine (1000 mg/m^2 day 1, 8, 15/28 days) for six months [12], or 5-fluorouracil (425 mg/m^2 and folinic acid 20 mg/m^2 day 1–5 every 28 days) for six months [13]. The combination of gemcitabine (1000 mg/m^2 day 1, 8, 15/28 days) and capecitabine (1660 mg/m^2/day 1 to 21/28 days) for six months presented longer survival [14]. Regimens based on FOLFIRINOX or gemcitabine in combination with albumin-bound paclitaxel are recommended to patients with borderline resectable lesions [15]. Chemoradiotherapy is another option for borderline resectable patients with microscopically positive margin of resection (R1), and locally advanced unresectable disease [16,17].

Patient's prognosis after resection could be predicted based on pathological parameters such as positive margins of resection, differentiation of tumor cells, lymph node status, etc. [18]. Therefore, an early detection of this type of cancer is crucial for successful treatment and to increase patient survival [19]. However, the only biomarker approved by the Food and Drug Administration (FDA) in PC is CA19-9 [20]. CA19-9 presents low specificity, so its utility has been questioned and its use is limited to predict recurrence after surgical resection [21]. Then, to better understand the poor prognosis of PC, further molecular studies are required [18].

P-element-induced wimpy testis (PIWI) proteins belong to the Argonaute (AGO) family and are expressed mainly in germline cells [22]. AGO proteins play an important role in the regulation of gene expression through complementary recognition of short RNAs, which guide them against their target genes [23]. In human, PIWI proteins consist of four members: PIWIL1, PIWIL2, PIWIL3, and PIWIL4 [24]. Specifically, PIWI proteins recognize and bind a type of non-coding small RNAs called piRNAs (PIWI-interacting RNAs), that constitutes the piRNA-induced silencing complex (piRISC). They have important roles in epigenetic regulation, the silencing of transposable elements, the protection of genome integrity, gametogenesis, and piRNA biogenesis [25].

In recent years, PIWI proteins have been linked to some of the hallmarks of cancer such as cell proliferation, the maintenance of genomic integrity, apoptosis evading, invasion, and metastasis [26,27]. This suggests that they could be used for cancer diagnosis and prognosis. The number of studies that show different expression patterns in healthy and tumor samples is increasing. In this context, an aberrant expression of PIWIL1 and PIWIL2 have been associated with different types of cancer and showed a variable prognostic and diagnostic potential [28]. The prognostic potential of PIWIL1 expression was previously evaluated in 56 PC samples at mRNA and protein levels. This study showed no impact on the survival of elevated PIWIL1 protein nor mRNA expression levels. However, altered mRNA expression (low or high expression compared to intermediate expression) presented poor prognosis only in the male population (p = 0.034) [29]. In respect to PIWIL2, the functional and clinical significance has not been reported in PC patients. Thus, the purpose of the present study is to evaluate the protein expression profile of PIWIL1 and PIWIL2 and assess the prognostic significance of these biomarkers in complete resected biliopancreatic tumors to guide subsequent adjuvant treatment decisions.

2. Experimental Section

2.1. Patients

A total of 190 biliopancreatic cancer patients who underwent surgery from 2006 to 2012 at the Surgery Department of University Hospital Clinico San Carlos were assessed for eligibility. Patients were followed-up to March 2019. Tumors were surgically resected and formalin-fixed and paraffin-embedded (FFPE) immediately for pathologic diagnosis. Tissue microarrays (TMA) were constructed with 182 available FFPE tumor samples. All the patients that presented positive margins of resection (R1) were excluded from the study (n = 53), resulting in 129 complete resected patients (R0). To assess survival analysis, only patients with available data of progression-free (n = 114) or overall survival (n = 117) were included in the study. At the end of the study, 45/114 (39%) patients did not progress, while 69/114 (61%) progressed on disease. Furthermore, 21/117 (18%) were alive, while 96/117 (82%) died at the study end. The tumor histology was reviewed by experienced pathologists. Since it is a retrospective study, PIWIL1 and PIWIL2 did not affect clinical decisions.

2.2. Immunohistochemistry

A tissue microarray was constructed for immunohistochemistry analysis and contained 364 cores (two cores per patient) using the MTA-1 tissue arrayer (Beecher Instruments, Tartu, Estonia). Each core (diameter, 1 mm) was punched from pre-selected tumor regions in paraffin-embedded tissues. Staining was conducted in 2-µm sections. Slides were deparaffinized by incubation at 60 °C for 10 min and incubated with PT-Link (Dako, Denmark) for 20 min at 95 °C in a high pH-buffered solution. To block endogenous peroxidase, holders were incubated with peroxidase blocking reagent (Dako, Denmark). Biopsies were incubated for 20 min with a 1:100 dilution of anti-PIWIL1 antibody (ab12337; Abcam, Cambridge, UK), 1:250 dilution of anti-PIWIL2 antibody (ab181340; Abcam, Cambridge, UK), 1:100 dilution of anti-hepatocyte nuclear factor (HNF)-4-alpha antibody (ab92378; Abcam Cambridge, UK), 1:20 dilution of anti-Mucin-17 (MUC17) antibody (ab122184; Abcam, Cambridge, UK), or 1:500 dilution of anti-pancreatic and duodenal homeobox 1 (PDX1) antibody (ab134150; Abcam, Cambridge, UK). Tissues were incubated with the appropriate anti-immunoglobulin horseradish peroxidase-conjugated polymer (EnVision, Dako, Denmark) to detect antigen–antibody reaction. All the antibodies and anti-Ig horseradish peroxidase-conjugated antibody presented high specificity, and no positiveness resulted from these antibodies individually. To determine immunohistochemistry conditions, different human tissues were used as a positive control according to The Human Protein Atlas (http://www.proteinatlas.org): Testis tissue for both anti-PIWIL1 and anti-PIWIL2 antibodies, small intestine tissue for the MUC17 antibody, human colon tissue for the HNF4A antibody, and human pancreatic tissue for the PDX1 antibody. Sections were then visualized with 3,3′-diaminobenzidine as a chromogen for 5 min and counterstained with haematoxylin. Photographs were taken with a stereo microscope (Leica DMi1, Wetzlar, Germany). To quantify the PIWIL1, PIWIL2, and MUC17 immunostaining, a semiquantitative HistoScore (Hscore) was calculated, and HNF4A and PDX1 immunostaining were categorized as positive or negative, since they are nuclear markers. The Hscore was determined by estimation of the percentage of positively stained cells with low, medium, or high intensity of staining, after applying a weighting factor to each estimate. The following formula was used: Hscore = (low%) × 1 + (medium%) × 2 + (high%) × 3, and the results ranged from 0 to 300. Quantification for each patient biopsy was calculated with the average of both cores by two independent researchers.

2.3. Statistical Analysis

The association between protein expression and survival, both progression-free and overall survival, was assessed with Kaplan–Meier curves, and analysis was performed with a log-rank test. Progression-free survival was defined as the interval between the dates of surgery and recurrence (local or distant). Overall survival was defined as the interval between the dates of surgery and patient

death or lost follow-up. The best cut-off point to identify low-risk or high-risk patients was determined by ROC (Receiver Operating Characteristics) curves for both progression-free (Area under the curve (AUC) = 0.862 for PIWIL1; AUC = 0.801 for PIWIL2) and overall survival (AUC = 0.764 for PIWIL1; AUC = 0.840 for PIWIL2). The Cox proportional hazards model was used to assess the hazard ratios and confidence intervals of both PIWIL1 and PIWIL2 expression, and clinicopathological variables of patients with only pancreatic origin. Thus, only statistically significant variables found in the univariate analysis were included in the multivariate analysis.

The association between PIWIL1 or PIWIL2 expression and clinicopathological variables was evaluated by Chi-square or Fisher exact tests.

To describe the association between PIWIL1 and PIWIL2 mRNA and the most significant factors associated to each of the molecular profiles of pancreatic cancer described by Bailey et al. [30], a 186-patient dataset from The Cancer Genome Atlas (TCGA) was analyzed using cBioPortal [31,32]. To validate previous results at the protein level, the Kolmogorov–Smirnov test was used to determine the normal distribution of PIWIL1, PIWIL2, and MUC17 Hscores. A Spearman test was used to evaluate the linear correlation between non-parametric variables (PIWIL1, PIWIL2, and MUC17); interpretation was performed according to Cohen et al. [33]. Positive or negative nuclear staining of HNF4A or PDX1 were associated to PIWIL1 or PIWIL2 with the Chi-square test. p-value ≤ 0.05 was considered statistically significant. Statistical analysis was performed with the IBM SPSS program, version 20.0.

2.4. Ethics Statement

All the human samples were kindly supplied by the BioBank of University Hospital Clinico San Carlos (B.0000725; PT17/0015/0040; ISCIII-FEDER). All the patients gave written informed consent for the use of their biological samples for research purposes. The institutional review board (IRB) of the University Hospital Clinico San Carlos evaluated the present study, granting approval on 10 March 2017 with approval number nº 17/091-E. Moreover, fundamental ethical principles promoted by Spain (LOPD 15/1999) and the European Union Fundamental Rights of the EU (2000/C364/01) were followed. In addition, all the patients' data were processed according to the Declaration of Helsinki (last revision 2013) and Spanish National Biomedical Research Law (14/2007, of 3 July).

3. Results

3.1. Patients Characteristics

Our cohort was well-balanced in terms of sex, and the median age of patients was 72 years (range 44 to 94 years). Pathologic diagnosis revealed the size of the resected tumors to be higher than 2 cm in 53% (n = 68) of cases. Tumors were stage I in 35% of cases (15% (n = 20) stage IA, and 20% (n = 26) stage IB); and stage II in 58% of cases (18% stage IIA (n = 23), and 40% (n = 51) stage IIB) according to the recommendations of the College of American Pathologists [34]. Most of the patients did not receive adjuvant treatment (59%, n = 76). Tumors presented as low grade in 82% (n = 106) of cases. Forty percent of patients (n = 51) showed lymph-node involvement, and most patients had vascular and neural invasion (33% (n = 43) and 55% (n = 71), respectively). Tumors were originated in the pancreas in 65% (n = 84), in the ampulla in 18% (n = 23), and in the bile duct in 15% (n = 20) of the cases. All the patients included in the study were completely resected, and thus presented negative surgical margins of resection (R0). An overview of the clinicopathological parameters of the patients is given in Table 1.

To verify whether the expression of PIWIL1 or PIWIL2 could be closely related to any of the clinicopathological characteristics registered in our study, a crosstab was performed thereafter (Table 2). In this analysis, PIWIL1 was associated significantly with gender (p = 0.035), where low levels of PIWIL1 are more often present in the male population, and also showed a high trend toward significance with pancreatic origin (p = 0.072). Low PIWIL2 expression had a statistically significant association with a higher T stage (p = 0.040). This result suggests that the lack of PIWIL2 expression exhibits

a deleterious effect on the patients analyzed. Furthermore, lower PIWIL2 expression exhibited a trend toward significance with other pathologic characteristics associated to tumor aggressiveness such as vascular invasion ($p = 0.068$), neural invasion ($p = 0.108$), tumor stage ($p = 0.111$), or lymph nodes involved ($p = 0.128$) (Table 2).

Table 1. Clinicopathological characteristics of complete resected biliopancreatic cancer patients recruited in the study.

Clinical Characteristics	*n*	%	Clinical Characteristics	*n*	%
Age			**Grade**		
<65 years	25	19	High	19	15
>65 years	104	81	Low	106	82
Gender			N/A	4	3
Male	63	49	**Vascular invasion**		
Female	66	51	No	76	59
Adjuvant treatment			Yes	43	33
No	76	59	N/A	10	8
Yes	24	19	**Neural invasion**		
N/A	29	22	No	48	37
Tumor origin			Yes	71	55
Pancreas	84	65	N/A	10	8
Ampulla	23	18	**pT**		
Bile duct	20	15	T1	30	23
N/A	2	2	T2	45	35
Size			T3	51	40
<2 cm	31	24	N/A	3	2
>2 cm	69	54	**Lymph nodes involved**		
N/A	29	22	No	70	54
Stage			Yes	51	40
IA	20	15	N/A	8	6
IB	26	20	**TOTAL**	129	100
IIA	23	18			
IIB	51	40			
N/A	9	7			

N: number of patients; N/A: Not available; cm: centimeters.

Table 2. Statistical association between P-element-induced wimpy testis 1 (PIWIL1) and P-element-induced wimpy testis 2 (PIWIL2) protein expression with clinico-pathological characteristics.

Parameters	PIWIL1 Low *n* (%)	PIWIL1 High *n* (%)	*p*-Value	PIWIL2 Low *n* (%)	PIWIL2 High *n* (%)	*p*-Value
Gender			0.035			0.390
Male	43 (33%)	20 (15%)		40 (31%)	23 (18%)	
Female	33 (26%)	33 (26%)		37 (29%)	29 (22%)	
Age			0.304			0.383
<65 years	17 (13%)	8 (6%)		13 (10%)	12 (9%)	
>65 years	59 (46%)	45 (35%)		64 (50%)	40 (31%)	
Stage			0.560			0.111
IA	9 (8%)	11 (9%)		7 (6%)	13 (11%)	
IB	17 (14%)	9 (8%)		15 (12%)	11 (9%)	
IIA	14 (11%)	9 (8%)		14 (12%)	9 (8%)	
IIB	30 (25%)	21 (17%)		34 (28%)	17 (14%)	
Adjuvant treatment			0.324			0.689
No	42 (42%)	34 (34%)		44 (44%)	32 (32%)	
Yes	16 (16%)	8 (8%)		15 (15%)	9 (9%)	
pT			0.960			0.040
T1	17 (13%)	13 (10%)		12 (10%)	18 (14%)	
T2	26 (21%)	19 (15%)		27 (21%)	18 (14%)	
T3	28 (22%)	23 (18%)		35 (28%)	16 (13%)	

Table 2. *Cont.*

Parameters	PIWIL1 Low *n* (%)	PIWIL1 High *n* (%)	*p*-Value	PIWIL2 Low *n* (%)	PIWIL2 High *n* (%)	*p*-Value
Size			0.536			0.968
<2 cm	15 (15%)	16 (16%)		19 (19%)	12 (12%)	
>2 cm	38 (38%)	31 (31%)		42 (42%)	27 (27%)	
Lymph nodes involved			0.978			0.128
No	41 (34%)	29 (24%)		37 (31%)	33 (27)	
Yes	30 (25%)	21 (17%)		34 (28%)	17 (14%)	
Vascular Invasion			0.893			0.068
No	45 (38%)	31 (26%)		40 (34%)	36 (30%)	
Yes	26 (22%)	17 (14%)		30 (25%)	13 (11%)	
Neural Invasion			0.891			0.108
No	29 (24%)	19 (16%)		24 (20%)	24 (20%)	
Yes	42 (35%)	29 (25%)		46 (39%)	25 (21%)	
Grade			0.142			0.703
Low	59 (47%)	47 (38%)		62 (50%)	44 (35%)	
High	14 (11%)	5 (4%)		12 (10%)	7 (5%)	
Tumor origin			0.072			0.197
Pancreas	67 (53%)	17 (13%)		54 (43%)	30 (24%)	
Bile duct	14 (11%)	6 (5%)		12 (9%)	8 (6%)	
Ampulla	13 (10%)	10 (8%)		10 (8%)	13 (10%)	

N: Number of patients; cm: centimeters.

3.2. PIWIL1 Expression has no Impact on Patient Survival

All the samples that stained positively for PIWIL1 exhibited a cytoplasmic expression pattern and some membrane localization, especially in some cases with high expression levels (Figure 1A left). In fact, PIWIL1 expression was also detected in the cytoplasm of some stroma cells, although all the cases with positive stained stroma cells showed stronger positiveness in tumor cells than in the stroma. Subsequently, tumor samples were divided into low or high expression of the PIWIL1 protein according to the ROC curve to associate its expression to survival (Figure 1B,C). Association between PIWIL1 and both progression-free and overall survival did not achieve statistical significance ($p = 0.311$ and $p = 0.166$, respectively).

3.3. PIWIL2 Expression Associated with Better Prognosis of Patients

Since PIWIL2 protein expression has not yet been determined by The Human Protein Atlas Project, we used a testis sample as a control to test the optimum concentration of antibody, as previously performed for PIWIL1 evaluation (Figure S1). PIWIL2 protein expression not only localized on the cytoplasm of tumor cells, but also weakly in the cytoplasm of stroma cells in those cases with high PIWIL2 expression levels (Figure 2A left). The association between PIWIL2 protein expression and outcome of patients was assessed. For this, patients were stratified into low-risk and high-risk according to a cut-off point determined by the ROC curve. Interestingly, the expression of PIWIL2 protein in tumor samples had a statistically significant association with progression-free survival ($p = 0.036$; Figure 2B). Indeed, patients with a high expression of PIWIL2 presented longer median progression-free survival (median = 28 months; 95% CI: 18–38 months) than patients with low PIWIL2 expression (median = 11 months; 95% CI: 7–15 months). Then, an association between PIWIL2 protein expression and overall survival was also assessed. PIWIL2 protein expression was associated with longer overall survival ($p = 0.012$; Figure 2C). Here, patients with high PIWIL2 expression presented longer overall survival (median = 32 months; 95% CI: 23–41 months), while patients with low expression of PIWIL2 showed shorter overall survival (median = 16 months; 95% CI: 8–24 months). These results suggest that low PIWIL2 expression is a negative variable for survival outcome.

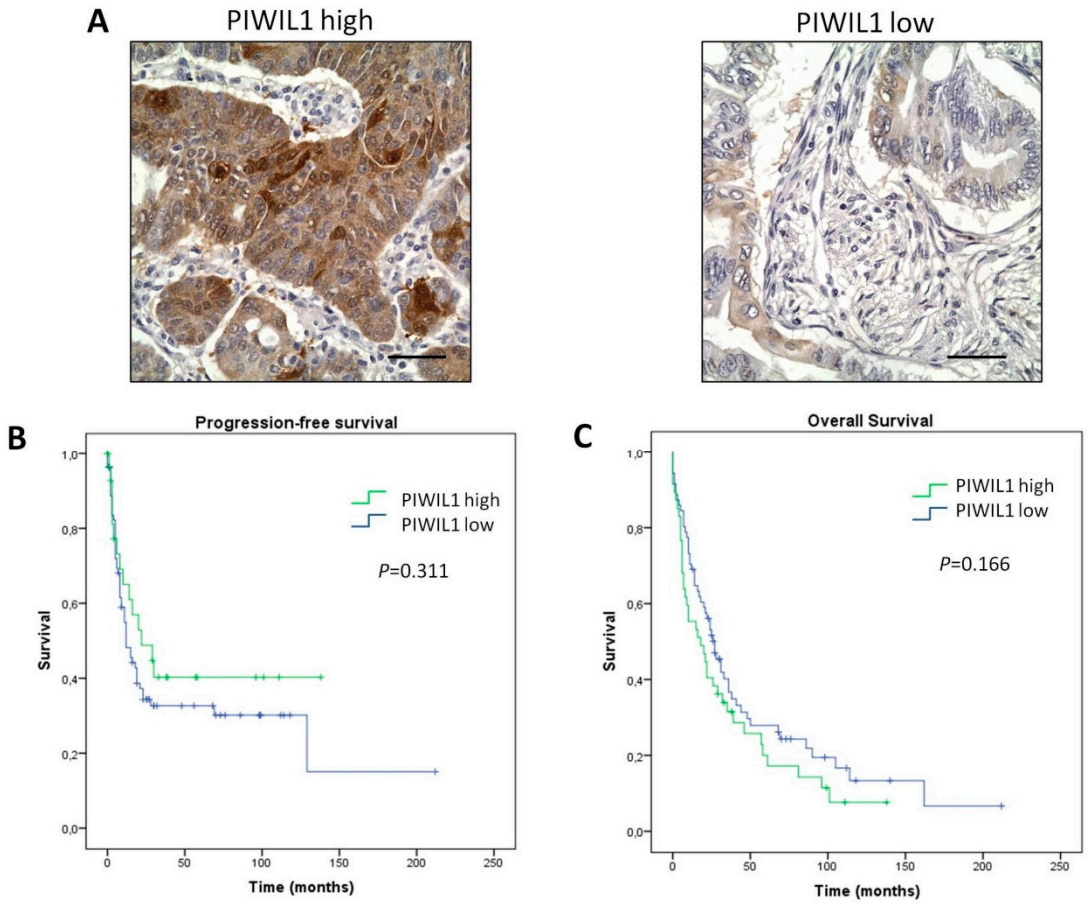

Figure 1. PIWIL1 expression has no impact on the outcome of biliopancreatic cancer patients. (**A**) Representative micrographs of high (left) and low (right) PIWIL1 expression tumors. (**B**,**C**) Kaplan–Meier curves for progression-free survival and overall survival analysis of patients, respectively. P-values were obtained by log-rank test. Scale bars: 50 μm.

Since all the biliopancreatic tumors included in the present study originated in the pancreas (n = 84), in the bile duct (n = 20), or in the ampulla of Vater (n = 23), we analyzed both the progression-free and overall survival of patients according to PIWIL2 expression stratified by their tumor origin (Figure 3). Interestigly, PIWIL2 expression associated with pancreatic origin in both progression-free survival and overall survival. Patients with pancreatic tumor origin that exhibited a high expression of PIWIL2 presented longer median progression-free survival (median = 29 months; 95% CI: 17–40 months) than patients with low PIWIL2 expression (median = 11 months; 95% CI: 7–14 months) (p = 0.029; Figure 3A-top). The overall survival of patients with pancreatic tumor origin with high PIWIL2 expression was longer (median = 32 months; 95% CI: 8–55 months) than patients with low expression of PIWIL2 (median = 16 months; 95% CI: 8–23 months) (p = 0.025; Figure 3B-top). The other tumor origins such as bile duct or ampulla were not associated with PIWIL2 expression for neither progression-free (Figure 3A—middle and bottom, respectively) nor overall survival (Figure 3B—middle and bottom, respectively). Therefore, this result supports the role of PIWIL2 as a prognostic biomarker in those biliopancreatic tumors that originated in the pancreas.

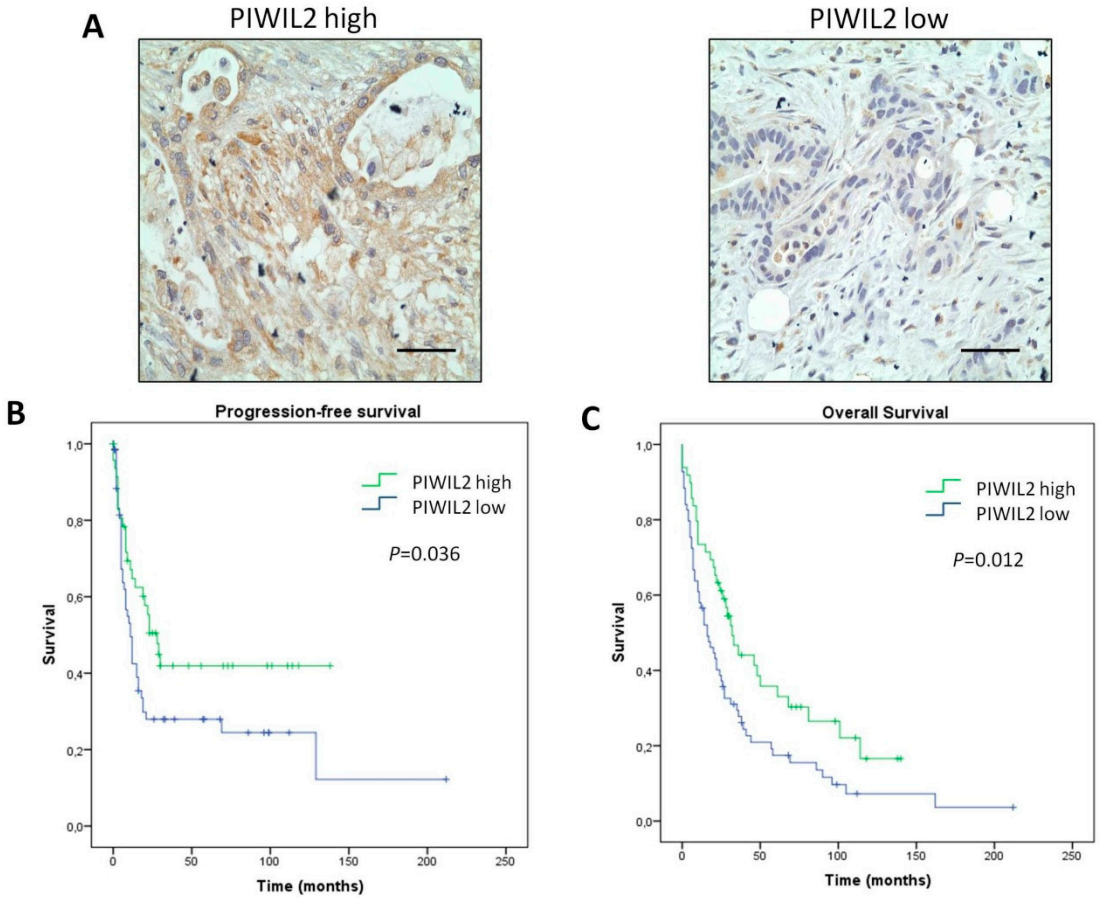

Figure 2. PIWIL2 expression predicts better prognosis in biliopancreatic cancer patients. (**A**) Representative micrographs of high-risk (left) and low-risk (right) PIWIL2 expression tumors. (**B**,**C**) Kaplan–Meier curves for progression-free survival and overall survival analysis of patients, respectively. P-values were obtained by log-rank test. Scale bars: 50 μm.

In order to compare the potential prognosis value of PIWIL2 expression with the other clinical variables, we performed a Cox proportional hazards model for both progression-free and overall survival of patients with only pancreatic tumor origin (Table 3). The univariate analysis for progression-free survival revealed that patients with a low expression of PIWIL2 showed a higher risk of recurrence after surgery compared to those with a high expression of PIWIL2 (hazard ratio, or HR = 1.788; 95% CI: 0.987–3.249). Although PIWIL2 did not raise significance to predict progression-free survival, our research found a high trend toward significance (p = 0.057). Here, the only significant variable associated with progression-free survival was the tumor stage—especially stage IIA, which presented the highest risk (HR = 3.568; 95% CI: 1.221–10.431; p = 0.020). On the other hand, the univariate analysis for overall survival revealed the potential of low levels of PIWIL2 to predict poor prognosis (HR = 1.832; 95% CI: 1.064–3.154; p = 0.029). Moreover, neural invasion appeared to be statistically associated with overall survival (HR = 1.819; 95% CI: 1.000–3.310; p = 0.050). Then, multivariate analysis between PIWIL2 expression and neural invasion as covariate revealed PIWIL2 as the unique statistically significant factor associated to overall survival (HR = 1.726; 95% CI: 0.946–3.154; p = 0.039) (Table 3). Thus, this result highlights the role of low expression of PIWIL2 as a detrimental factor in tumors with pancreatic origin.

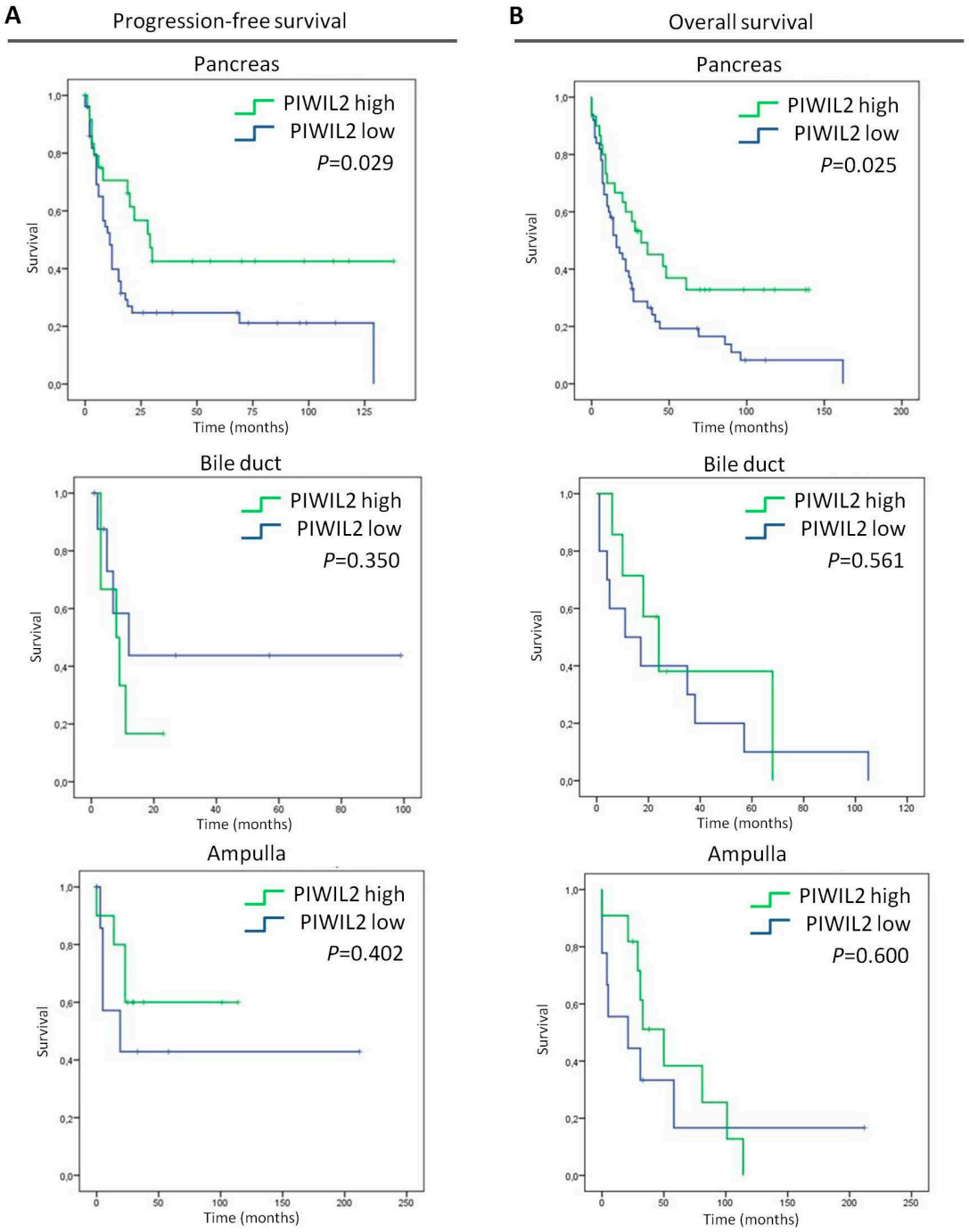

Figure 3. Survival analyses of patients according to their tumor origin and PIWIL2 expression. (**A**) Kaplan–Meier plots of progression-free survival, and (**B**) Kaplan–Meier plots of the overall survival of patients with a high expression of PIWIL2 (green lines) and patients with a low expression of PIWIL2 (blue lines). *p*-values were obtained by the log-rank test.

Table 3. Univariate and multivariate proportional hazard model of PIWIL2 and other clinical variables for progression-free and overall survival of those patients with pancreatic tumor origin.

	Univariate PFS (95% CI)				Univariate OS (95% CI)			
	HR	**Lower**	**Upper**	***p*-Value**	**HR**	**Lower**	**Upper**	***p*-Value**
Age (<65 years vs. >65 years)	1.359	0.695	2.657	0.370	1.320	0.715	2.439	0.375
Gender (Male vs. Female)	1.420	0.814	2.478	0.217	1.083	0.657	1.785	0.756
Adjuvant treatment (Yes vs. No)	1.168	0.603	2.260	0.645	1.321	0.781	2.428	0.371
Size (<2 cm vs. >2 cm)	1.792	0.633	5.073	0.272	1.163	0.519	2.603	0.714
Stage				0.050				0.148
IA	1.000				1.000			
IB	1.367	0.466	4.008	0.569	1.161	0.466	2.890	0.748
IIA	3.568	1.221	10.431	0.020	2.491	0.975	6.362	0.056
IIB	2.393	0.900	6.364	0.080	1.733	0.743	4.040	0.203
Grade (low vs. high)	1.156	0.492	2.716	0.740	1.138	0.540	2.395	0.734
Lymph nodes involved (No vs. Yes)	1.457	0.817	2.599	0.202	1.266	0.746	2.150	0.382
Vascular invasion (No vs. Yes)	1.466	0.809	2.658	0.208	1.427	0.827	2.464	0.202
Neural invasion (No vs. Yes)	1.815	0.936	3.521	0.078	1.819	1.000	3.310	0.050
pT (I vs. II/III)	1.690	0.841	3.396	0.141	1.505	0.797	2.841	0.207
PIWIL2 (high vs. low)	1.788	0.987	3.249	0.057	1.832	1.064	3.154	0.029
	Multivariate PFS (95% CI)				**Multivariate OS (95% CI)**			
Neural invasion (No vs. Yes)					1.726	0.946	3.151	0.075
PIWIL2 (high vs. low)					1.813	1.030	3.189	0.039

PFS: progression-free survival; OS: Overall survival; HR: hazard ratio; CI: confidence interval; vs.: versus; cm: centimeters.

3.4. PIWIL1 and PIWIL2 Expression is Associated to Progenitor Molecular Subtype of Pancreatic Cancer

Given our previous results related to patient outcome, we wonder whether PIWIL1 or PIWIL2 would be associated to any of the four described molecular subtypes of pancreatic cancer [30]. Then, mRNA expression of the most significant factors associated with each molecular subtype of pancreatic cancer of 186 pancreatic cancer patients from a TGCA dataset was correlated with PIWIL1 or PIWIL2 mRNA expression levels (Figure 4). Interestingly, we found a positive moderate correlation between PIWIL1 or PIWIL2 and most of the genes that characterize the progenitor molecular subtype. Here, PWIIL1 mRNA correlated moderately with MUC17 (r = 0.385), MUC13 (r = 0.381), HNF4g (r = 0.320), HNF4a (r = 0.282), MUC1 (r = 0.254) or HNF1a (r = 0.228). PIWIL2 mRNA exhibited a positive moderate correlation with HNF4g (r = 0.480), HNF4a (r = 0.398), MUC17 (r = 0.339), PDX1 (r = 0.332) or HNF1b (r = 0.291) (Figure 4).

Since these findings are observed at the mRNA level, we decided to validate this correlation at the protein level. We chose three different factors; within these, mRNA correlated with piwil1 and piwil2 with a higher correlation coefficient and that associated to different molecular mechanisms, according to three criteria. Firstly, as HNF4G is not expressed in pancreatic tissue according to The Human Protein Altas, we selected HNF4A (hepatocyte nuclear factor 4) because of its crucial role in pancreatic β-cells development and since its mutations cause a type of maturity-onset diabetes of the young. Secondly, we selected MUC17 (Mucin-17), which is a type of mucin overexpressed in pancreatic tumor cell lines and tumor tissues compared with the normal pancreas. Finally, we selected PDX1 (pancreatic and duodenal homeobox 1), which is necessary for pancreatic development, including β-cell maturation.

Consequently, we stained our 186 tumor cohort to evaluate these markers (Figure S2). Curiously, PIWIL2 protein expression correlated positively with MUC17 (r = 0.225; p = 0.002). Furthermore, PIWIL2 associated significantly with HNF4A (p = 0.016), and PIWIL1 associated significantly with PDX1 (p = 0.036). Hence, statistical analyses of both mRNA and the protein level support the association between PIWIL1 and PIWIL2 with the progenitor molecular subtype of PC.

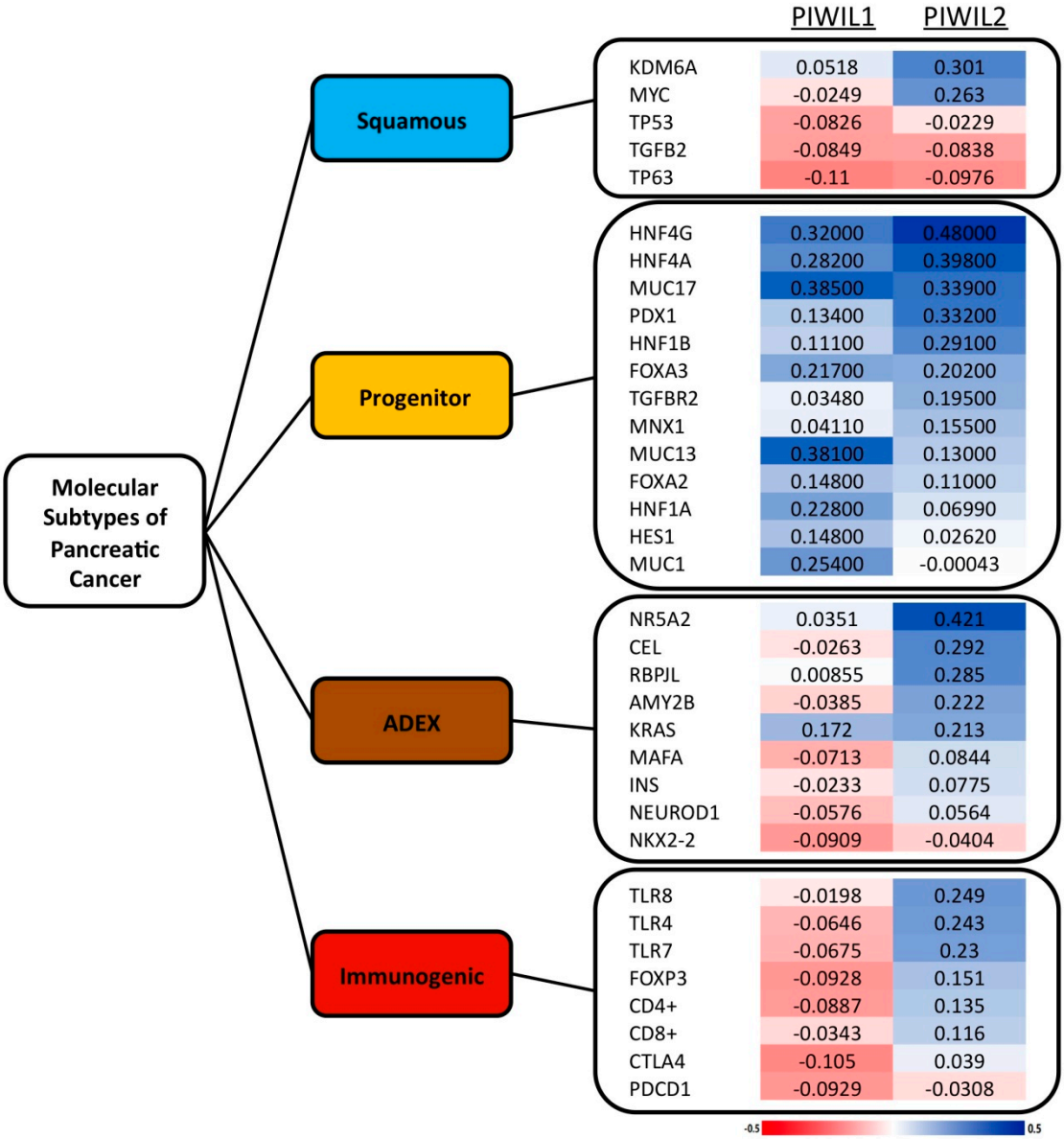

Figure 4. Expression of piwil1 or piwil2 is associated with most significant factors of the progenitor molecular subtype of pancreatic cancer at the mRNA level. The figure shows the Spearman correlation coefficients between the most relevant genes for each molecular subtype versus piwil1 or piwil2 mRNA expression. The red charts correspond to negative coefficients and the blue charts correspond to positive coefficients, in a scale ranging from −0.5 to 0.5.

4. Discussion

Despite all the scientific advances in PC, patients' outcome is still poor, and incidence of this disease is increasing; therefore, we need new molecular targets to bring promising approaches. In this regard, PIWI proteins provide new insights to address the therapeutic challenges of PC. The *PIWI* gene family is a novel class of highly conserved genes that encodes basic proteins with a high homology [35]. PIWI proteins incorporate both a RNA endonuclease catalytic domain and an anchor site for the 5′ phosphate of the RNA guide strand [36], which confers their ability to recognize small interfering RNA known as PIWI-interacting RNAs [37–39]. PIWI proteins are involved in stem cell division, gametogenesis, germline specification, and RNA silencing [35,40,41]. In humans, PIWI proteins are necessary for spermatogenesis [35] and the maintenance of hematopoietic stem cells [42]. Therefore, the conserved functions of PIWI proteins in stem cell maintenance suggest their potential role in

tumorigenesis with a low grade of differentiation. The first tumor where PIWI proteins were studied was a testis tumor that originated from germ and non-germ cells [43].

In this clinical research, we have focused on the study of PIWIL1 and PIWIL2 protein expression to dissect their prognostic value in PC and assist physicians in patient management. PIWI proteins have been associated to several neoplasias after being described for the first time in a testis tumor [26]. The *PIWIL1* gene is located closed to the extreme of the long arm of chromosome 12q24.33, and its expression was associated to gastric cancer and precancerous development with an expression pattern similar to Ki67 [44]. In addition, PIWIL1 has been described to exhibit a poor prognostic value in soft-tissue sarcoma [45,46], esophageal squamous cell carcinoma [47], colorectal cancer [48–50], gliomas [51], human hepatocellular carcinoma [52,53], gastric cancer [54,55], lung cancer [56], gynecological cancers [57–59], renal cell carcinoma [60,61], and non-small cell lung cancer [62]. In contrast, the overexpression of PIWIL1 seems to have a beneficial effect in chronic myeloid leukemia, since it is able to inhibit the growth and migration of tumor cells [63] and increases sensitivity to daunomycin [64]. Moreover, PIWIL1 is considered a potential target for treatment design in glioblastoma [65], hepatocellular carcinoma [66], and lung cancer [67]. Only one study reported the prognosis significance of PIWIL1 in PC determined by mRNA and protein levels [29]. As observed by authors, neither PIWIL1 protein nor mRNA expression levels had an impact on survival. However, in this study, the altered mRNA expression (high or low) presented shorter survival than those patients with intermediate levels of PIWIL1 mRNA ($p = 0.034$) [29]. From our point of view, intermediate mRNA expression should be rather limited at clinical practice. For this reason, we focused our study on protein expression levels evaluated by immunohistochemical staining. Similarly to previously reported results, PIWIL1 protein expression does not have enough statistical power to be considered a prognostic biomarker, although it exhibited an association with male patients and a trend toward significance with pancreatic tumor origin.

On the other hand, we have also evaluated the prognostic value of PIWIL2 in our patient cohort. The *PIWIL2* gene is localized in chromosome 8p21.3, and its expression has also been associated with tumor development of some gynecological cancers [57,59], renal cell carcinoma [60,61], breast cancer [68], gastric cancer [54], and colorectal cancer [69]. Since The Human Protein Atlas Project only provides PIWIL2 mRNA expression, we described for the first time the protein expression profile of PIWIL2 in human tumor samples by using human testis tissues to determine the best antibody concentration. Consequently, survival analyses in our patient cohort according to PIWIL2 protein expression revealed the lack of PIWIL2 as a negative event in prognosis that reduces both progression-free and overall survival. Furthermore, the survival analysis performed with patients stratified by tumor origin revealed the prognosis significance of PIWIL2 expression and its potential value to predict the outcome of patients with tumors that originated in the pancreas. This effect was also supported by the Cox multivariate analysis for overall survival, where PIWIL2 expression remained the only significant molecular event ahead of neural invasion. Moreover, the statistical association between low expression levels of PIWIL2 and higher T status, and a high trend toward significance with vascular invasion, neural invasion, and higher stages support the role of low expression levels of PIWIL2 as a detrimental effect on the progression and development of such tumors. Our result was in accordance with the findings of Litwin et al., which reported decreased PIWIL2 mRNA expression in colorectal cancer tissues compared to untransformed tissues ($p < 0.001$) [70]. PIWIL2 protein expression has also been found to be downregulated in non-small cell lung cancer samples in comparison to the normal tissue ($p < 0.001$) [62]. In addition, PIWIL2 mRNA levels have been statistically significant lower in breast carcinoma samples compared to normal breast tissues ($p < 0.001$) [70]. Consequently, the tumorigenic effect of PIWIL2 expression seems to be rather controversial and remains unclear. In this concern, the epigenetic modulation of PIWI proteins' expression plays a crucial role and could justify their ambivalent role in tumorigenesis through the upregulation of DNA methyltransferases [71].

PIWI proteins regulate several molecular pathways through key mediators in different neoplasias. Here, we describe that most of the significant factors associated to the progenitor molecular subtype

of PC have a positive correlation with PIWIL1 or PIWIL2 at the mRNA level. Moreover, the protein expression of PIWIL2 correlated positively with MUC17 and associated significantly with HNF4A at the protein level, while PIWIL1 expression associated significantly with PDX1 protein expression. This fact supports the link between these two PIWI proteins and the progenitor molecular subtype of PC, which is involved in early pancreatic endoderm development and related to maturity onset diabetes of the young [30].

Besides the link between PIWI proteins and progenitor subtype, these proteins have been associated to several genes involved in the cell cycle regulation, apoptosis, proliferation, and migration of tumor cells. For example, PIWIL1 is able to regulate OCT4, which is a factor associated to poor prognostic and metastasic disease in colorectal cancer [70]. PIWIL1 also regulates apoptosis and cell cycle progression through P21, Cyclin D1, BCL-2 and BAX, and migration through expression of MMP-2 and MMP-9 in glioma cells [72]. In contrast, PIWIL1 expression downregulates MMP-2 and MMP-9 and inhibits the proliferation and migration ability of chronic myeloid leukemia cells [63]. In gastric cancer, *PIWIL1* has been related to *OCK2*, *ZNF503*, *PDE4D*, *ABL1*, *ABL2*, *LPAR1*, *SMAD2*, *WASF3*, and *DACH1* genes [73], and it has exhibited a regulation activity of epithelial-to-mesenchymal transition in endometrial cancer [74]. PIWIL2 regulates BCL-XL and STAT3, and its downregulation both suppresses protein expression triggering apoptosis cascade [75], and enhances cisplatin sensitivity [76]. Interestingly, Chen et al. suggested that the ectopic expression of PIWIL2 contributes to the development and proliferation of precancerous stem cells, which have the potential for both benign and malignant differentiation [77]. Furthermore, a positive correlation between PIWIL2 and the undifferentiated cell marker SOX2 have been observed in colorectal cancer tissues [70].

5. Conclusions

This study shows the differential role of PIWIL1 and PIWIL2 in pancreatic cancer. On the one hand, the expression of PIWIL1 did not associate to pancreatic cancer prognosis; thus, further research is needed to dissect the role of PIWIL1 in pancreatic cancer progression. On the other hand, the results presented here support the role of PIWIL2 protein expression as a prognostic biomarker in pancreatic cancer, and suggest a link between PIWIL2 expression and the progenitor molecular subtype of pancreatic cancer. However, PIWIL2 function in cancer initiation and development is rather controversial, and remains unclear. Therefore, future translational research might be focused on those piRNAs regulated by PIWIL2. The identification of piRNAs, in both solid tumors and serum samples, and their function will provide new insights of PIWI proteins and their role as diagnostic, prognostic, or predictive biomarkers.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/8/9/1275/s1, Figure S1: Immunohistochemical staining for PIWIL1 and PIWIL2 protein expression at different antibodies concentrations in human testis tissue; Figure S2: Immunohistochemical staining for HNF4A, MUC17 and PDX1 protein expression.

Author Contributions: Conceptualization: J.M.-U. and J.G.-F.; methodology: J.M.-U., W.L., N.G.-C.; validation: W.L.; formal analysis: J.M.-U., W.L., N.G.-C. and M.J.F.-A.; investigation: J.M.-U., W.L., N.G.-C., and J.G.-F.; resources: L.O.-M., S.G.-B., E.P.-A., L.D.-V.; data curation: J.M.-U., W.L. and J.G.-F.; writing—original draft preparation: J.M.-U.; writing—review and editing: J.M.-U. and J.G.-F.; visualization: J.M.-U., W.L., N.G.-C., M.J.F.-A., L.O.-M., S.G.-B., E.P.-A., L.D.-V. and J.G.-F.; supervision: J.M.-U. and J.G.-F.; project management: J.M.-U.; funding acquisition: J.G.-F.

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SUPPLEMENTARY INFORMATION

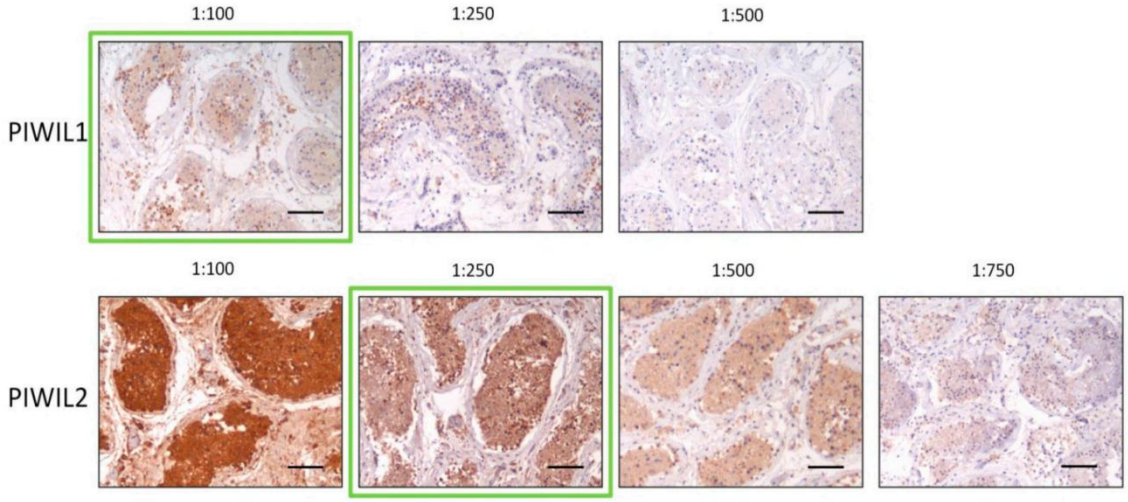

Figure S1. Immunohistochemical staining for PIWIL1 and PIWIL2 protein expression at different antibodies concentrations in human testis tissue. 1:100, 1:250, and 1:500 dilutions for anti-PIWIL1 antibody; and 1:100, 1:250, 1:500, and 1:750 dilutions for anti-PIWIL2 antibody were assessed. Green boxes show the optimal working dilution for each antibody. Scale bars represent 100 µm.

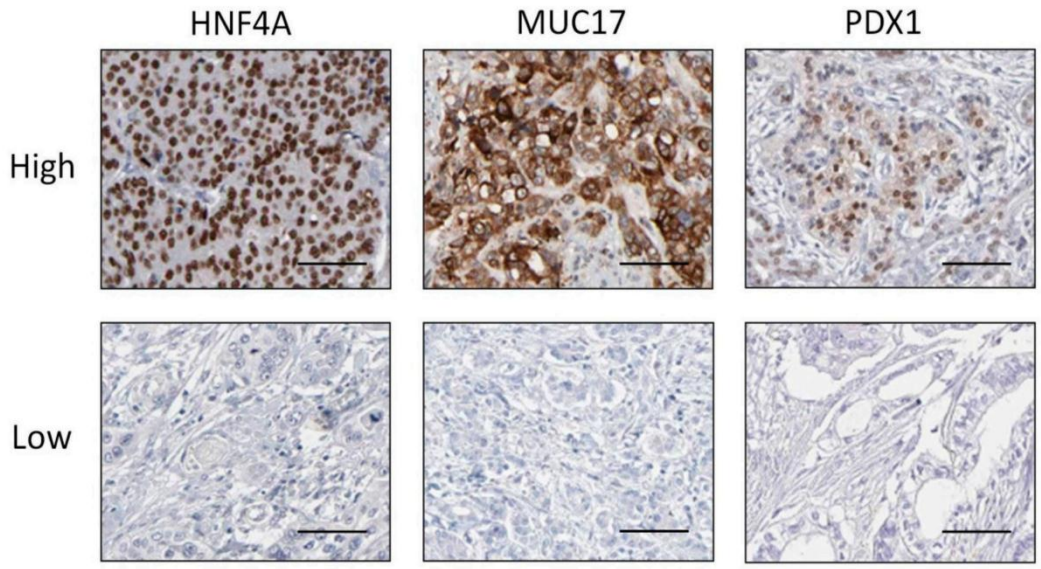

Figure S2. Immunohistochemical staining for hepatocyte nuclear factor 4A (HNF4A), Mucin-17 (MUC17), and pancreatic and duodenal homeobox 1 (PDX1) protein expression. The figure shows representative images of high (up) and low (down) expression tumors for each antibody. Scale bars represent 50 µm.

ARTÍCULO 2: The Clinical Significance of PIWIL3 and PIWIL4 Expression in Pancreatic Cancer.

PIWIL1 y PIWIL2 se habían evaluado en el anterior artículo; sin embargo, el papel de PIWIL3 y PIWIL4 en la carcinogénesis de cáncer de páncrea era desconocida y en otros tumores era bastante controvertido.

En este trabajo, evaluamos la expresión de PIWIL1, PIWIL2, PIWIL3 y PIWIL4 en líneas celulares derivadas de CaPa y en una línea celular no tumoral. Aquí, mostramos una expresión diferencial en líneas celulares tumorales y no tumorales de PIWIL3 y PIWIL4. Posteriormente, los experimentos funcionales con la desregulación de PIWIL3 y/o PIWIL4 revelaron una disminución en la proporción de motilidad de las líneas celulares tumorales y no tumorales a través de la regulación a la baja de los factores mesenquimales en pro de los factores epiteliales. También observamos que el silenciamiento de PIWIL3 y/o PIWIL4 altera el fenotipo indiferenciado y aumenta la toxicidad del fármaco en líneas celulares tanto derivadas de tumores como sanas. Finalmente, la evaluación de PIWIL3 y PIWIL4 en muestras de pacientes mostró que los bajos niveles de expresión de la proteína PIWIL4 presentaban un mal pronóstico.

Aportación Personal al trabajo:

En este trabajo mi aportación se centró en realizar todos los experimentos, me encargué de la evaluación de la expresión de PIWIL1, PIWIL2, PIWIL3 y PIWIL4 en líneas celulares por western blot e inmunohistoquímica, el silenciamiento de PIWIL3 y PIWIL4 y llevar a cabo los experimentos funcionales de migración e invasión por cierre de herida y Transwell, así como el mantenimiento de los cultivos celulares y colonosferas. También me encargué de llevar a cabo la inmunohistoquímica de los TMA (microarrays de tejidos) de pacientes y los análisis estadísticos de los resultados. Finalmente, colaboré en la redacción del artículo y de la posterior revisión del mismo tras las correcciones oportunas de mis directores de tesis.

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The Clinical Significance of PIWIL3 and PIWIL4 Expression in Pancreatic Cancer

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Abstract: P-element-induced wimpy testis (PIWI) proteins have been described in several cancers. PIWIL1 and PIWIL2 have been recently evaluated in pancreatic cancer, and elevated expression of PIWIL2 conferred longer survival to patients. However, PIWIL3's and PIWIL4's role in carcinogenesis is rather controversial, and their clinical implication in pancreatic cancer has not yet been investigated. In the present study, we evaluated PIWIL1, PIWIL2, PIWIL3 and PIWIL4 expression in pancreatic cancer-derived cell lines and in one non-tumor cell line as healthy control. Here, we show a differential expression in tumor and non-tumor cell lines of PIWIL3 and PIWIL4. Subsequently, functional experiments with PIWIL3 and/or PIWIL4 knockdown revealed a decrease in the motility ratio of tumor and non-tumor cell lines through downregulation of mesenchymal factors in pro of epithelial factors. We also observed that PIWIL3 and/or PIWIL4 silencing impaired undifferentiated phenotype and enhanced drug toxicity in both tumor- and non-tumor-derived cell lines. Finally, PIWIL3 and PIWIL4 evaluation in human pancreatic cancer samples showed that patients with low levels of PIWIL4 protein expression presented poor prognosis. Therefore, PIWIL3 and PIWIL4 proteins may play crucial roles to keep pancreatic cell homeostasis not only in tumors but also in healthy tissues.

Keywords: PIWI proteins; PIWIL3; PIWIL4; pancreatic cancer; EMT; chemoresistance; motility; HNF4A; survival

1. Introduction

Pancreatic cancer (PC) has arisen as one of the tumors with higher incidence in developed countries. Indeed, the incidence of PC is expected to be higher than breast, prostate or colorectal cancers and to reach the second cause of cancer-related death by 2030 [1]. The 5-year survival rate is 50% when tumors are <2 cm in size and close to 100% for tumors <1 cm [2]; unfortunately, PC is normally asymptomatic, and it is often diagnosed when the tumor has metastasized to distant organs [3]. Adjuvant treatment for complete resected patients (R0) is usually based on Gemcitabine [4], or 5-fluorouracil for six months [5]. Regimens based on Gemcitabine in combination with Nanoalbumin bound-Paclitaxel (Nab-Paclitaxel)

is recommended to patients with advanced disease [6]. Nevertheless, PC develops multi-pathways chemoresistance as a result of the interaction between tumor cells, cancer stem cells and the tumor microenvironment [7].

P-element-induced wimpy testis (PIWI) proteins belong to the Argonaute (AGO) family and have been firstly discovered in germline cells [8]. Based on their protein sequence, eight members of the Argonaute family have been classified into two subfamilies: the PIWI subfamily (PIWIL1, PIWIL2, PIWIL3 and PIWIL4) and the AGO subfamily (AGO1, AGO2, AGO3 and AGO4) [9]. The AGO family regulates gene expression through complementary recognition and guidance of short RNAs against their target genes [10]. Recently, it has been reported how PIWI proteins are expressed during the epigenetic remodeling and meiosis of the germline [11]. They also recognize and bind a unique type of non-coding small RNAs called piRNAs (PIWI-interacting RNAs), which constitutes the so-called piRNA-induced silencing complex (piRISC). PIWI proteins have an important role in epigenetic regulation, silencing of transposable elements, protection of genome integrity, gametogenesis and piRNA biogenesis [12]. Indeed, the expression of PIWI proteins promotes some of the hallmarks of cancer such as cell proliferation, genomic integrity, apoptosis, invasion and metastasis [13]. Therefore, an increasing number of studies report their differential expression patterns between healthy and tumor samples and how their modulation affects the behavior of tumor cells. PIWIL1 downregulation drastically reduces the proliferation, invasion and migration of hepatocellular carcinoma cells [14]. Other studies describe how PIWIL1 downregulation in sarcoma inhibits cell growth and allows cell differentiation and support the idea that PIWIL1 tumorigenic activity is due to its ability to regulate DNA hypermethylation [15]. Downregulation of PIWIL1 suppresses cell proliferation, migration and invasion of gastric cancer and lung cancer cells [16–18]. These studies sustain the oncogenic features of PIWIL1 and support the idea that PIWIL1 could be used as a target for anticancer therapies. In contrast, other reports showed that overexpression of PIWIL1 decreases proliferation and migration of chronic myeloid leukemia cells through inhibition of expression of matrix metalloproteinase-2 and -9 [19]. Our group has recently described the prognostic role of PIWIL1 and PIWIL2 protein expression in PC, especially PIWIL2 protein, which exhibited higher prognostic potential to predict longer progression-free survival ($p = 0.029$) and longer overall survival ($p = 0.025$). Furthermore, we provided new insight into the link between PIWIL1 and PIWIL2 with the progenitor molecular subtype of PC [20].

PIWIL3 is expressed in stage III epithelial ovarian cancer in both primary tumor and metastatic tissues compared with their adjacent normal tissues ($p < 0.01$), and the expression is higher in the metastatic foci [21]. PIWIL3 is also considered a prognostic biomarker of breast cancer since its upregulation was significantly associated to a short progression-free survival ($p = 0.01$) and a poor overall survival ($p = 0.02$) [22]. Furthermore, PIWIL3 seems to play a crucial role in melanoma progression, and its expression is higher with the higher tumor stage [23]. In gastrointestinal cancers, expression of PIWIL3 was also higher in tumors compared with their paired untransformed tissues [24]. Furthermore, upregulation of PIWIL3 increases proliferation, migration and invasion of gastric cancer cells [24]. In contrast, PIWIL3 seems to play a protective effect due to its overexpression-reduced proliferation, migration and invasion of glioma cells in vitro and decreased tumor size in vivo [25].

The role of PIWIL4 involves chromatin modifications in human somatic cells [26], and it is able to process precursor hairpins to generate several miRNAs in the absence of the endoribonuclease DICER [27]. The lack of PIWIL4 could derive to the development of type 2 diabetes since its downregulation in pancreatic beta cells resulted in defective insulin secretion [28]. However, its function in tumorigenesis is rather controversial. On the one hand, high expression of PIWIL4 is found in tumor tissues of colorectal cancer [29], cervical cancer [30], gastric cancer [31] and primary and metastatic foci of ovarian cancer [21] compared with their adjacent tissues. Its downregulation not only enhanced significantly the apoptotic effect of treatment in Leydig cell tumor [32] but also apoptosis, migration and invasion of breast cancer cells in vitro [33,34]. In hepatocellular carcinoma, the nuclear expression of PIWIL4 together with PIWIL2 has been found to confer worse outcome [35]. On the other hand, other studies have reported that low PIWIL4 expression was significantly associated

with a worse prognosis in hepatocellular carcinoma [36], soft tissue sarcoma [37], non-small cell lung cancer [38] and renal cell carcinoma [39]. Low levels of PIWIL4 were also found in hepatocellular carcinoma tissues [36] and in other tumors like breast tumors [22] and non-small cell lung cancer [38] compared to the non-cancerous tissues. Moreover, the lack of PIWIL4 expression caused by CpG island hypermethylation has also been found in testicular tumors [40].

Since PIWIL3 and PIWIL4 expression has not been studied in PC and the functions of PIWI proteins in cancer seem to be rather controversial, we have evaluated the role of PIWIL3 and PIWIL4 expression in pancreatic cells and dissect their prognostic potential in PC.

2. Experimental Section

2.1. Cell Lines and Cell Culture

The human PC-derived cell lines PANC 04.03(ATCC ref: CRL-2555), PL45(ATCC ref: CRL-2558), BxPC-3(ATCC ref: CRL-1687) and one non-tumor human pancreatic ductal epithelial cell line hTERT-HPNE (ATCC ref: CRL-4023) were purchased and cultured under American Type Culture Collection (ATCC) recommendations. RWP1 and PANC-1 were kindly provided by Dr. Fatima Gebauer (CRG, Barcelona, Spain). RWP1, PANC-1 cells were routinely grown in RPMI supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (P/S). All cell lines were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Patient Samples

We evaluated the prognostic potential of PIWIL3 and PIWIL4 in a training set and in a validation set of PC samples with tissue microarrays (TMA). TMA of the training set was performed with 44 formalin-fixed, paraffin-embedded tumor samples from BioBank of University Hospital Fundacion Jimenez Diaz—Universidad Autonoma de Madrid (PT13/0010/0012), and the TMA for validation set was constructed with 182 available formalin-fixed and paraffin-embedded tumor samples from BioBank of University Hospital Clinico San Carlos (B.0000725; PT17/0015/0040; ISCIII-FEDER). (Detailed descriptions of all experimental procedures are provided in Supplementary Information 1: Materials and Methods)

3. Results

3.1. PIWIL3 and PIWIL4 Are Overexpressed in Non-Tumor and Tumor-Derived Cell Lines

All human PIWI proteins were evaluated by Western blot and by immunohistochemistry (IHC) in a panel of five PC-derived cell lines: four from duct-adenocarcinoma differentiation (BxPC-3, Panc04.03, PL45 and RWP1), and one from epithelioid-carcinoma differentiation (PANC-1). Moreover, PIWI proteins were determined in one non-tumor cell line developed from human pancreatic duct transduced with a retroviral expression vector containing the human *TERT* gene (hTERT-HPNE) (Figure 1A,B).

Protein expression was compared with the expression of human testis as positive control. PIWIL1 and PIWIL2 showed very scarce expression in all pancreatic cell lines, not only in the tumor-derived but also in the non-tumor cell lines. PIWIL1 expression in all cell lines was not detected by WB (Figure 1A), although it could be visualized in some cells of BxPc-3 or Panc04.03 by IHC (Figure 1B). Expression levels of PIWIL2 were unnoticeable by both techniques (Figure 1A,B). In contrast, PIWIL3 and PIWIL4 showed overexpression in almost all tumor-derived cell lines, and in the non-tumor pancreatic cell line compared to control (Figure 1A,B). Both PIWIL3 and PIWIL4 exhibited a clear cytoplasmic expression pattern with some nuclear staining (Figure 1B). Panc04.03 was the only PC-derived cell line with the lowest expression levels of PIWIL3 or PIWIL4 (Figure 1A,B). Since PIWIL3 and PIWIL4 are expressed in the immortalized non-tumor pancreatic cell line, we cannot conclude that PIWIL3 or PIWIL4 could act as an oncogene. Then, we wondered whether PIWIL3 or

PIWIL4 take part in other mechanisms and which is the response of cells after PIWIL3 or PIWIL4 downregulation in the absence of PIWIL1 and PIWIL2. To this aim, we downregulated PIWIL3 and/or PIWIL4 with two different validated siRNA sequences. The highest expression levels have been shown in two pancreatic ductal adenocarcinoma-derived cell lines (PL45 and RWP1) (Figure 1C,D) and the non-tumor pancreatic cell line (hTERT-HPNE) (Figure 1E). As PL45 showed almost five-fold PIWIL3 expression levels compared with control, and two independent combinations with two different siRNA were necessary to downregulate PIWIL3 (Figure 1C). We also decided to evaluate PIWIL3 or PIWIL4 downregulation on hTERT-HPNE by IHC rather than by Western blot due to the low cellularity that exhibited this cell line. Here, we found that maximum PIWIL3 or PIWIL4 downregulation was achieved later in both tumor cell lines than in non-tumor cell line. Higher PIWIL3 or PIWIL4 downregulation in both tumor cell lines was achieved between the fifth/sixth days (Figure 1C,D) compared with the second day obtained in the non-tumor cell line (Figure 1E).

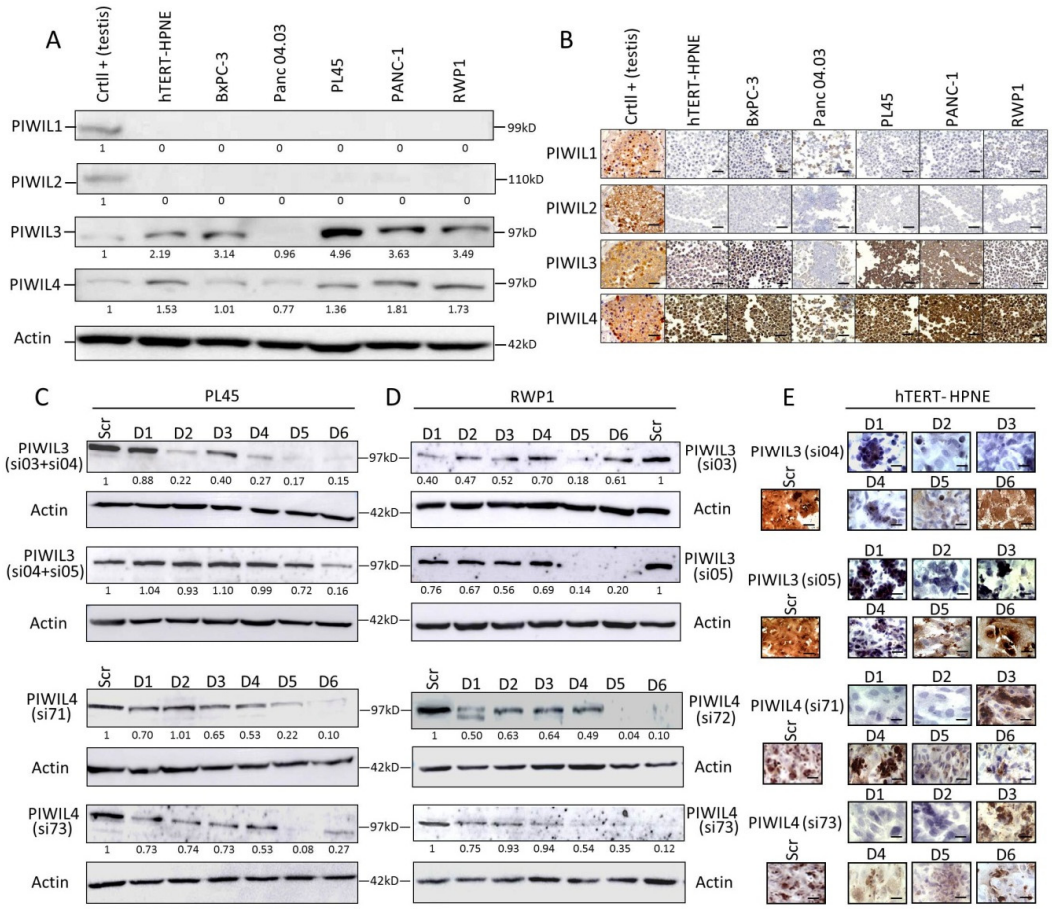

Figure 1. P-element-induced wimpy testis (PIWI) proteins present differential expression in pancreatic cancer (PC), and a late downregulation of PIWIL3 or PIWIL4 in tumor cell lines was found compared to non-tumor cell line. (**A**) Western blot analysis, and (**B**) representative micrographs of immunohistochemical staining of a panel of five human PC-derived cell lines and one non-tumor pancreatic cell line (hTERT-HPNE). A human testis tissue was used as positive control. Two independent downregulations of PIWIL3 (top) and PIWIL4 (bottom) were performed to carry out functional experiments with PL45 (**C**), RWP1 (**D**) and hTERT-HPNE (**E**). Crtl: control. kDa: kilodalton. Scr: Scramble. D1–6: Days 1–6. PIWIL3/Actin or PIWIL4/Actin ratio is represented under each protein band. Scale bar: 50 μm.

3.2. PIWIL3 and/or PIWIL4 Are Necessary for Cell Motility of Both Non-Tumor and Tumor-Derived Cell Lines

Since one of the characteristics of PC is its ability to migrate and metastasize to distant organs, we evaluated the role of PIWIL3 or PIWIL4 in cell motility. Here, we performed functional experiments with two different tumor-derived cell lines and one non-tumor cell line. Interestingly, wound healing assay showed a delay in the motility ratio in all cell lines, normal and tumoral, after PIWIL3 and/or PIWIL4 silencing (Figure 2A).

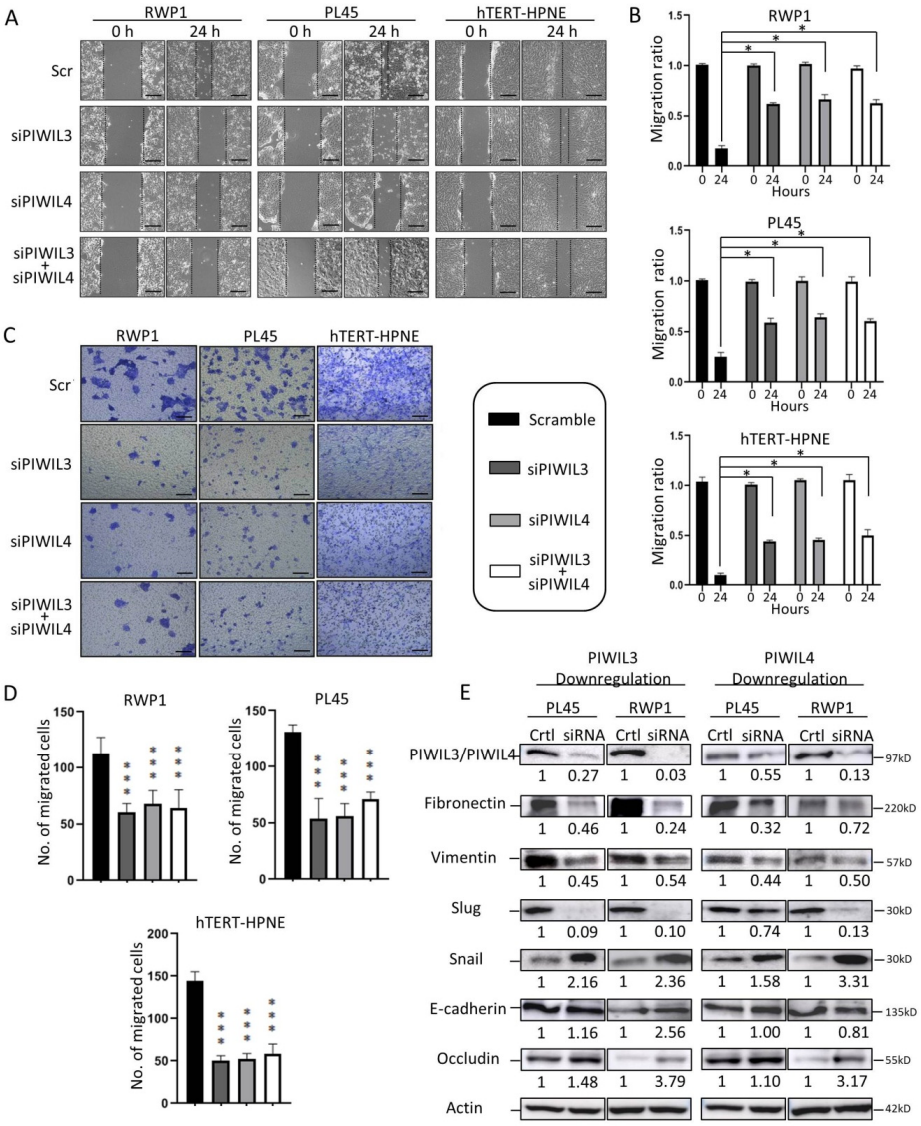

Figure 2. Downregulation of PIWIL3 and/or PIWIL4 decreased motility of PC and non-tumor cell lines through regulation of epithelial-to-mesenchymal transition (EMT). (**A**) Micrographs of wound healing assay showed reduced cell motility after PIWIL3 and/or PIWIL4 silencing in both PC-derived cell lines and in the non-tumor pancreatic-derived cell line. Representative images have been taken at 0 and 24 h after scratching. Broken lines indicate migration heads. (**B**) Statistical analyses of the motility ratio for each cell line according to PIWIL3 and/or PIWIL4 silencing. (**C**) Representative images from Boyden chamber assay of different cell lines taken at 24 h after seeding. (**D**) Statistical analyses of the number of migrated cells for each cell line according to PIWIL3 and/or PIWIL4 silencing. (**E**) Western blot for the expression of PIWIL3 (left) or PIWIL4 (right), Fibronectin, Vimentin, Slug, E-Cadherin and Occludin in PL45 and RWP1 after PIWIL3 or PIWIL4 silencing. The ratio of each protein/Actin ratio is represented under each protein band. Color-coding for each protein downregulation is indicated in the legend box. kDa: kilodalton. Scale bar: 50 µm. *$p < 0.05$; ***$p < 0.001$.

Statistical analyses compared to control revealed a significant reduction in the motility ratio of all cell lines downregulated for PIWIL3 or PIWIL4 individually or in combination ($p < 0.05$) (Figure 2B). To verify our previous results, a Boyden chamber assay was performed as previously described by Chen [41]. Although all cell lines and scrambles were cultured with the same chemotactic agent (20% FBS), the number of migrating cells decreased significantly after individual PIWIL3 and/or PIWIL4 knockdown alone or in combination compared to scramble ($p < 0.001$) (Figure 2C,D). Interestingly, this fact was not only observed in tumor cell lines but also in the normal cell line, which also decreased its motility after PIWIL3 and/or PIWIL4 downregulation. These results suggest that PIWIL3 and PIWIL4 not only modulate invasiveness of tumor cells but also motility of normal cells, which could impair wound healing processes of adult healthy tissues.

To further study how PIWIL3 and PIWIL4 affect cell motility, we evaluated the expression of different markers involved in epithelial-to-mesenchymal transition (EMT). Interestingly, the mesenchymal proteins Fibronectin and Vimentin reduced their expression after PIWIL3 or PIWIL4 downregulation (Figure 2E). Transition factor Slug highly reduced its protein level after PIWIL3 or PIWIL4 downregulation (Figure 2E). Moreover, epithelial markers like Occludin increased its expression after PIWIL3 or PIWIL4 knockdown in both cell lines, while E-Cadherin raised its protein levels only after PIWIL3 silencing (Figure 2E). These results highlight the role of PIWIL3/PIWIL4 in cell motility and wound healing of pancreatic cells through regulation of EMT factors. Taking into consideration that downregulation of PIWIL3 or PIWIL4 reverses EMT of normal cell line, the modulation of these two proteins could affect adult tissue reconstruction after trauma, toxic treatments or inflammatory processes.

3.3. Downregulation of PIWIL3 and/or PIWIL4 Impairs Undifferentiated Phenotype

Following with functional experiments with PIWIL3 and/or PIWIL4 downregulation, we evaluated the ability of both tumor and non-tumor pancreatic derived cell lines to form pancreatic spheres in stem cell enrichment culture media (Figure 3A).

PL45 was not able to dedifferentiate, and to the best of our knowledge, no detailed research reached PL45 dedifferentiation. The spheres observed from scramble controls ranged from 2 to 4 µm of diameter and formed between 10 and 20 spheres per 10,000 seeded cells. Non-tumor cell line presented the lowest number of spheres and the lowest sphere diameter in control conditions. Remarkably, we observed that PIWIL3 and/or PIWIL4 knockdown dropped drastically the number and diameter of spheres of tumor cell line RWP1 ($p < 0.001$) (Figure 3B). However, the same effect was observed on the non-tumor cell line, hTERT-HPNE, not only in the number of spheres ($p < 0.001$) but also in their diameter ($p < 0.05$) (Figure 3C). These results suggest the role of PIWIL3 and PIWIL4 in the maintenance of undifferentiated phenotype of pancreatic cells; however, it seems not to be only necessary for neoplastic cells, but also for normal cells differentiation. These results hamper the clinical use of PIWIL3 or PIWIL4 modulation in PC patients because it may disrupt the dedifferentiation mechanism not only of tumor cells but also of other healthy tissues and could lead to a severe medical condition for patients.

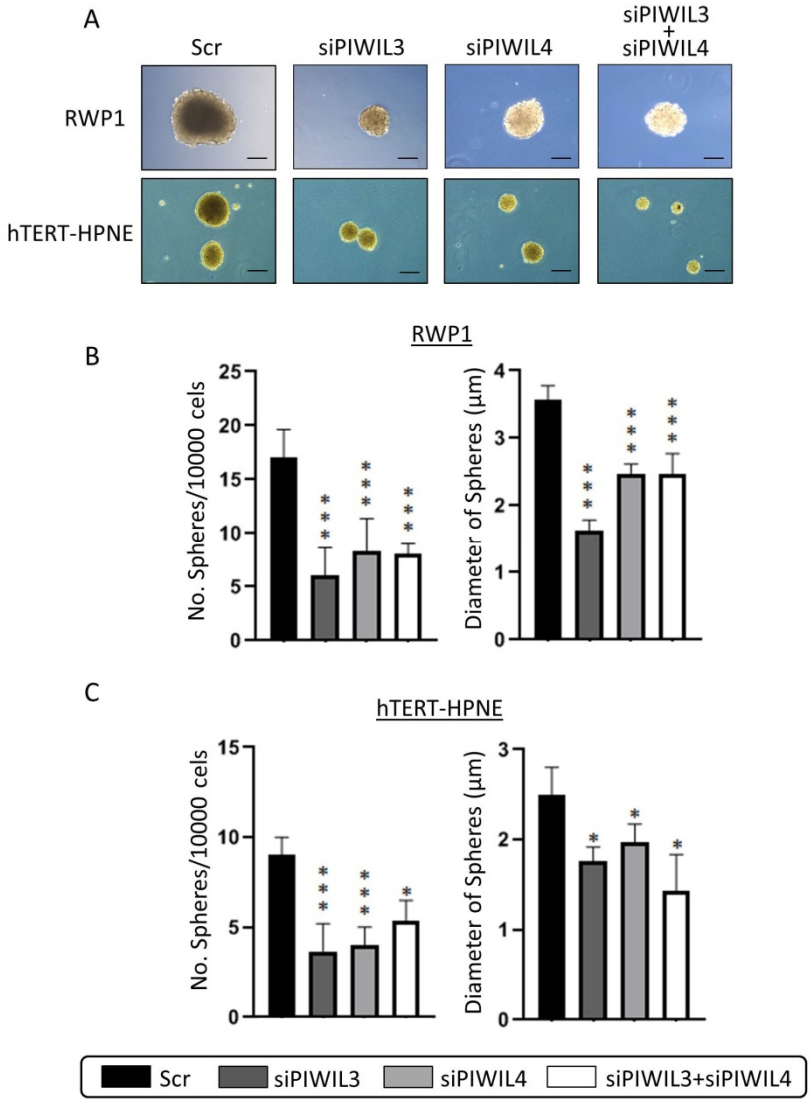

Figure 3. PIWIL3 and PIWIL4 impair undifferentiated phenotype. (**A**) Representative micrographs of undifferentiated pancreatic spheres derived from RWP1 and hTERT-HPNE transfected with siRNA for PIWIL3 (siPIWIL3) or PIWIL4 (siPIWIL4) downregulation individually or in combination. (**B**) Statistical analyses of number and diameter of spheres according to PIWIL3 and/or PIWIL4 downregulation of RWP1 cell line. (**C**) Statistical analyses of number and diameter of spheres according to PIWIL3 and/or PIWIL4 downregulation of the non-tumor hTERT-HPNE cell line. Color-coding for each protein downregulation is indicated in the legend box. Scr: scramble. Scale bar: 50 µm. * $p < 0.05$; *** $p < 0.001$.

3.4. PIWIL3 and PIWIL4 Downregulation Potentiate the Cytotoxic Effect of Chemotherapies

Gemcitabine is one of the gold standard adjuvant treatments for PC management, alone or in combination with Nab-Paclitaxel. Therefore, we wondered whether PIWIL3 and/or PIWIL4 regulate response to these chemotherapies. To evaluate the cytotoxicity of these two factors, tumor and normal cell lines were treated with Gemcitabine or Nab-Paclitaxel individually or in combination after PIWIL3 and/or PIWIL4 knockdown. Then, logarithmically growing tumor-derived cell lines, RWP1 and PL45, and normal cell line, hTERT-HPNE, were treated with previously determined IC_{50} doses of Gemcitabine or Nab-Paclitaxel (Supplementary file). To determine doses for treatment combination for each cell line, IC_{25} dose of Nab-Paclitaxel was set due to its high toxicity, and different concentrations of Gemcitabine were tested to achieve 50% of cell death as previously reported by Awasthi N. et al. [42]. Individual protein downregulation was not enough to achieve an effect, and PIWIL3 and PIWIL4 double

downregulation were necessary to decrease significantly cell viability of RWP1 after single treatments ($p = 0.023$ for Gemcitabine; $p = 0.038$ for Nab-Paclitaxel). PIWIL4 downregulation per se achieved a significant effect on the combined treatment ($p = 0.038$); although, double downregulation achieved the maximum effect ($p = 0.001$) (Figure 4A). In contrast, neither PIWIL3 nor PIWIL4 knockdown affected cytotoxicity of PL45 cell line, neither with individual treatments nor in combination (Figure 4B).

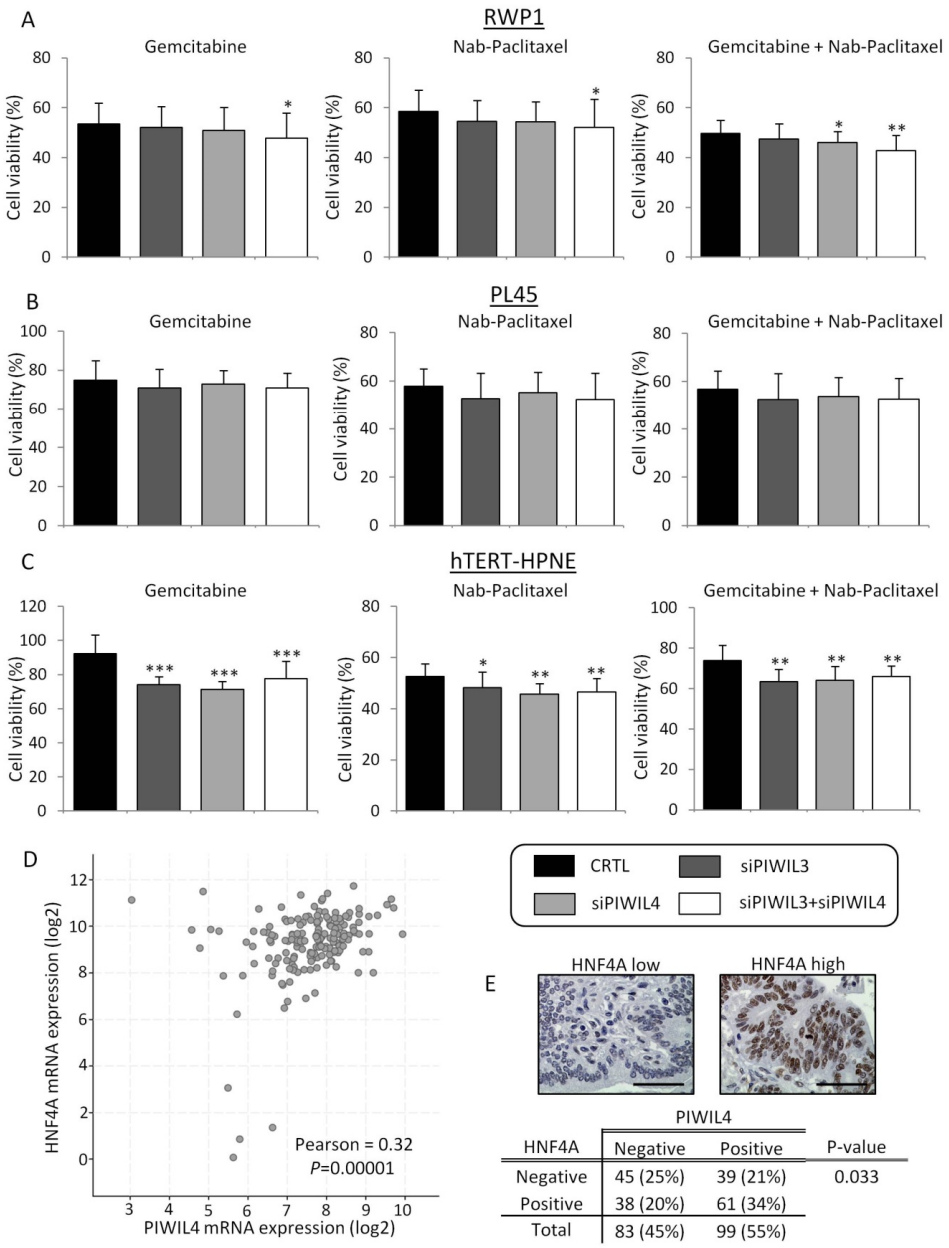

Figure 4. PIWIL3 and PIWIL4 downregulation potentiates the cytotoxic effect of chemotherapy. (**A**) Cell viability analyses after PIWIL3 and/or PIWIL4 silencing according to Gemcitabine (left) or Nab-Paclitaxel (center) individual treatments or in combination (right) of RWP1 cell line, PL45 (**B**) and hTERT-HPNE (**C**) cell lines. (**D**) Scatterplot and statistical analysis of HNF4A mRNA expression (y axis) and PIWIL4 mRNA expression (x axis) of 178 patient cohort from The Cancer Genome Atlas (TCGA). (**E**) Representative micrographs of HNF4A low expression (top-left) and high expression top-right). Statistical association between HNF4A and PIWIL4 protein expression of 182 PC samples (bottom). Color-coding for each protein downregulation is indicated in the legend box. Scale bars: 50 μm. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

On the other hand, non-tumor cell line hTERT-HPNE initially presented a complete resistance to Gemcitabine; then, functional experiments were performed with the highest concentration of Gemcitabine tested (250 μM). This concentration was 42,000 times higher than IC_{50} concentration of Gemcitabine for RWP1 and 700 times higher than IC_{50} concentration of Gemcitabine for PL45. Furthermore, IC_{50} dose of Nab-Paclitaxel for non-tumor cell line (235 μM), which was 21 times higher than IC_{50} dose of Nab-Paclitaxel for RWP1 and 1.6 times higher than for PL45. Interestingly, the highest effect of all treatments was observed in the non-tumor derived cell line. Indeed, PIWIL3 and/or PIWIL4 silencing overcame Gemcitabine resistance of the non-tumor cell line ($p < 0.001$), and significantly increased the other treatment effects ($p = 0.003$ for Nab-Paclitaxel; $p = 0.001$ for Gemcitabine + Nab-Paclitaxel) (Figure 4C). Therefore, these results support PIWIL3 and PIWIL4 as crucial factors in chemoresistance of PC tumor cells and in the toxicity of normal cells. However, from a clinical point of view, depletion of PIWIL3 or PIWIL4 proteins with target therapies should be done with great care due to the potential high toxicity and adverse events that they could bring to PC patients.

In order to dissect one of the underlying mechanisms whereby PIWIL3 or PIWIL4 expression confers chemoresistance, we evaluated the link between these two proteins and hENT1, which is responsible for Gemcitabine uptake and effect on cells [43]. For this, we used 178 available expression profile data from a 186-patient dataset from The Cancer Genome Atlas (TCGA, Firehose Legacy), and statistical correlation was assessed using cBioPortal [44,45]. In this first attempt, piwil3 or piwil4 showed no correlation with hEnt1 at mRNA level ($p = 0.26$ and $p = 0.19$, respectively). Another factor that drives cytotoxicity of tumor cells is HNF4A. It has been previously described to be a negative regulator of hENT1 and necessary for cell proliferation and drug resistance in PC [46]. Then, we assessed the correlation between piwil3 or piwil4 and hnf4a; however, piwil3 mRNA expression did not show any connection with hnf4a at the mRNA level ($p = 0.36$). Interestingly, mRNA analysis showed a moderate positive correlation between piwil4 and hnf4a ($r = 0.32$; $p = 0.00001$) (Figure 4D). To validate this result, we stained by IHC 182 PC patient samples with anti-HNF4A antibody. HNF4A exhibited a clear nuclear staining and a marked differential expression pattern between samples (Figure 4E, top). The statistical analysis revealed a link between PIWIL4 and HNF4A at the protein level in patient samples ($p = 0.033$)(Figure 4E, bottom). We also assessed an association between PIWIL3 and HNF4A at the protein level. Although no association was found, statistical analysis revealed a high trend towards significance ($p = 0.080$). These results highlight a connection between PIWIL3 and PIWIL4 with HNF4A factor, which could explain a feasible mechanism of chemoresistance of PC cells and cytotoxicity of normal cells.

3.5. Low Expression of PIWIL4 Is a Poor Prognosis Factor of Pancreatic Cancer Patients

To study the prognostic potential of PIWIL3 or PIWIL4 in PC, we evaluated their protein expression levels in a cohort composed of 44 patients from Fundacion Jimenez Diaz Hospital. To assess the survival analysis all samples with positive margins of resection (R1) were excluded from the study ($n = 7$ patients) (Table 1).

Immunohistochemical staining of patient samples showed differential expression levels of PIWIL3 and PIWIL4. All the samples that stained positively for PIWIL3 exhibited a cytoplasmic localization, especially in those cases with high PIWIL3 expression (Figure 5A). The expression pattern of PIWIL4 was limited to cytoplasm and cell membrane of tumor cells, and no positive nuclear staining was found (Figure 5B). Survival analyses were assessed with this data set. Nevertheless, neither PIWIL3 nor PIWIL4 associated significantly with progression-free or overall survival of PC patients (Figure 5C,D). However, although statistical analyses revealed no significant association between these PIWI proteins and prognosis, we found that patients with low expression levels of PIWIL3 or PIWIL4 presented shorter progression-free and overall survival than high levels of both proteins. The mean progression-free survival of patients with low PIWIL3 expression was 17 months (95% CI = 7–27 months), while the mean time-to-progression of high PIWIL3 expression was 30 months (95% CI = 6–54 months) (Figure 5C, top). Concerning overall survival, patients with low PIWIL3 expression exhibited a mean survival of

37 months (95% CI = 22–53 months), and those with high PIWIL3 expression lived a mean of 62 months (95% CI = 33–90 months) (Figure 5C, bottom). Similarly, low PIWIL4 expression presented shorter mean progression-free and overall survival than high-expression patients. The mean progression-free survival of patients with low PIWIL4 expression was 19 months (95% CI = 6–31 months), while the mean time-to-progression of high PIWIL4 expression was 23 months (95% CI = 8–39 months) (Figure 5D, top). Furthermore, patients with low PIWIL4 expression presented shorter overall survival (mean = 39 months; 95% CI = 23–56 months) than patients with high PIWIL4 expression (mean = 56 months; 95% CI = 30–82 months) (Figure 5D, bottom).

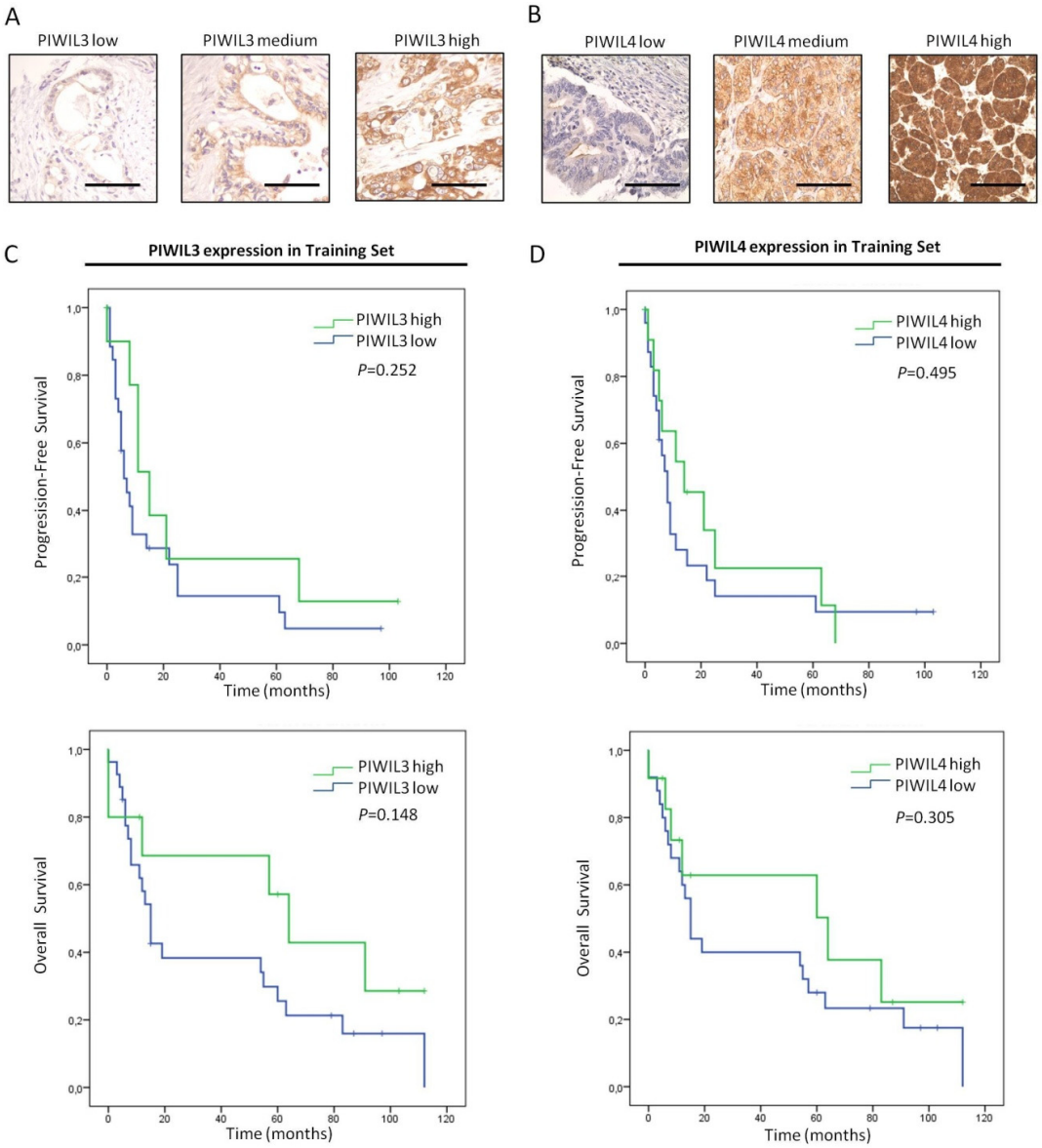

Figure 5. Prognostic impact of PIWIL3 or PIWIL4 in PC patients from the training set. (**A**) Representative micrographs of tumors with low (left), intermediate (middle) and high PIWIL3 expression (right). (**B**) Representative micrographs of tumors with low (left), intermediate (middle) and high PIWIL4 expression levels (right). (**C**) Kaplan–Meier curves according to PIWIL3 protein expression for both progression-free (top) and overall survival (bottom). (**D**) Kaplan–Meier curves according to PIWIL4 protein expression for both progression-free (top) and overall survival (bottom). *p*-values were obtained by log-rank test. Scale bars: 50 μm.

Table 1. Clinico-pathologic characteristics of completed resected R0 pancreatic cancer patients from the training set.

Clinical Characteristics	*N*	%	Clinical Characteristics	*N*	%
Age			Neural invasion		
<65 years	16	43	No	12	32
>65 years	21	57	Yes	25	68
Gender			Lymph nodes involved		
Male	21	57	N0	14	38
Female	16	43	N1	23	62
Size			Adjuvant treatment		
<2 cm	20	54	No	21	57
>2 cm	17	46	Yes	14	38
Stage			N/A	2	5
I	9	24	pT		
II	28	76	T1	6	16
Grade			T2	5	14
High	30	81	T3	26	70
Low	7	19	N/A	3	2
Vascular invasion					
No	12	32	Total	37	100
Yes	25	68			

N: number of patients; N/A: not available; cm: centimeters.

One of the possible reasons that may justify the lack of statistical significance of these analyses could be the limited sample size of the study. Therefore, we evaluated the expression of PIWIL3 and PIWIL4 in a larger cohort composed of 182 patients samples from Clinico San Carlos Hospital. As before, all samples with positive margins of resection were excluded from the study (n = 54 patients) (Table 2).

Table 2. Clinico-pathologic characteristics of complete resected R0 pancreatic cancer patients from the validation set.

Clinical Characteristics	*N*	%	Clinical Characteristics	*N*	%
Age			Grade		
<65 years	25	20	High	19	15
>65 years	103	80	Low	105	82
Gender			N/A	4	3
Male	63	49	Vascular invasion		
Female	65	51	No	75	59
Diabetes Mellitus			Yes	43	33
No	88	69	N/A	10	8
Yes	33	26	Neural invasion		
N/A	7	5	No	47	37
Adjuvant treatment			Yes	71	55
No	75	58	N/A	10	8
Yes	24	19	pT		
N/A	29	23	T1	30	23
Size			T2	44	35
<2 cm	31	24	T3	51	40
>2 cm	69	54	N/A	3	2
N/A	28	22	Lymph nodes involved		
Stage			N0	70	55
I	46	36	N1	51	40
II	74	58	N/A	7	5
N/A	8	6	Total	128	100

N: number of patients; N/A: not available; cm: centimeters.

We assessed survival analyses with patients with available data of progression-free survival (n = 113) or overall survival (n = 118). Here, PIWIL3 expression did not associate either with progression-free survival (p = 0.214) or overall survival (p = 0.337) (Figure 6A,B). Thus, these results led us to exclude PIWIL3 expression as a prognostic biomarker for PC. Interestingly, those PC patients with low expression of PIWIL4 presented not only a shorter progression-free survival (p = 0.002) but also a shorter overall survival (p < 0.001) than patients with high expression levels (Figure 6C,D). Here, patients with low PIWIL4 expression showed a mean progression-free survival of 31 months (95% CI = 20–41 months), while patients with high PIWIL4 expression presented a mean progression-free survival of 75 months (95% CI = 54–96 months) (Figure 6C). Overall survival of patients with low PIWIL4 expression presented a mean of 29 months (95% CI = 21–37 months), while that of patients with high PIWIL4 expression was significantly longer with a mean of 68 months (95% CI = 46–89 months) (Figure 6D).

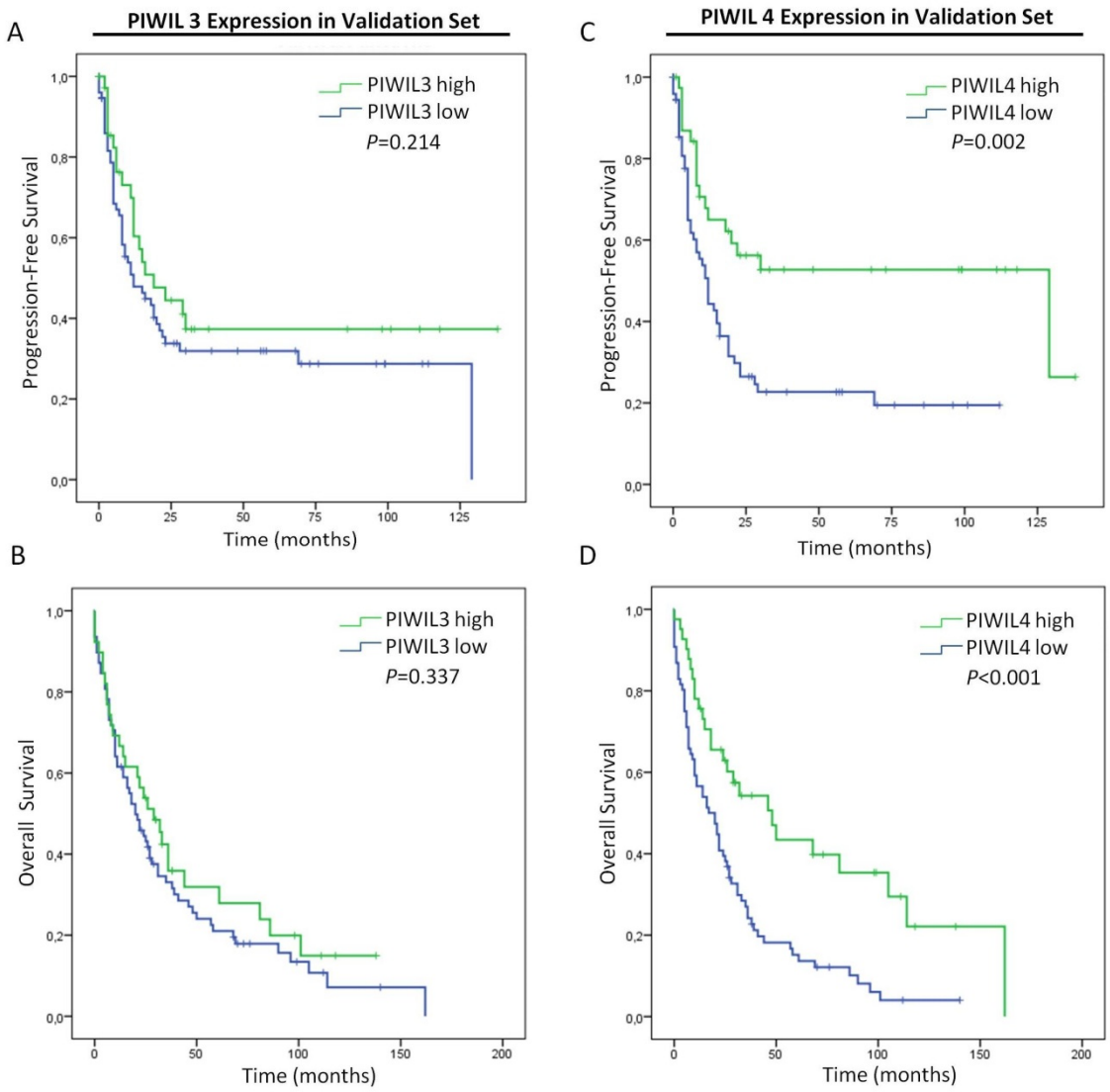

Figure 6. Prognostic impact of PIWIL3 or PIWIL4 in PC patients from the validation set. (**A**) Kaplan–Meier curves according to PIWIL3 protein expression for progression-free survival. (**B**) Kaplan–Meier curves according to PIWIL3 protein expression for overall survival. (**C**) Kaplan–Meier curves according to PIWIL4 protein expression for progression-free survival. (**D**) Kaplan–Meier curves according to PIWIL4 protein expression for overall survival. p-values were obtained by log-rank test.

In order to validate the prognosis potential of PIWIL4 expression with respect to other clinico-pathological characteristics, we performed a Cox proportional hazards model for both progression-free and overall survival of patients (Table 3). The univariate analysis for progression-free survival revealed that patients with a low expression of PIWIL4 showed a higher risk of recurrence after surgery compared with patients with high expression (hazard ratio (HR) = 1.979; 95% CI: 1.178–3.325; $p = 0.010$). As survival curves confirmed previously, PIWIL3 did not raise significance to predict progression-free survival ($p = 0.227$). Other pathological characteristics that associated significantly with high risk of progression in the univariate analysis were tumor size (HR = 3.023; 95% CI: 1.413–6.465; $p = 0.004$), T stage (HR = 1.682; 95% CI: 1.033–2.738; $p = 0.037$), tumor stage (HR = 1.866; 95% CI: 1.105–3.151; $p = 0.020$) and neural invasion (HR = 1.757; 95% CI: 1.027–3.007; $p = 0.040$). In the multivariate analysis, low PIWIL4 expression remained statistically significant for a higher risk of progression (HR = 2.036; 95% CI: 1.025–4.044; $p = 0.042$) together with tumor size (HR = 3.095; 95% CI: 1.237–7.744; $p = 0.016$). Univariate analyses for overall survival also revealed low expression of PIWIL4 as a high risk factor (HR = 2.093; 95% CI: 1.344–3.260; $p = 0.001$). Other clinico-pathologic characteristics that associated significantly with shorter overall survival were T stage (HR = 1.679; 95% CI: 1.110–2.540; $p = 0.014$), tumor stage (HR = 1.795; 95% CI: 1.148–2.807; $p = 0.010$), lymph nodes positive (HR = 1.573; 95% CI: 1.025–2.414; $p = 0.038$) and neural invasion (HR = 1.658; 95% CI: 1.060–2.593; $p = 0.027$). However, the only clinical variable that associated significantly with reduced overall survival in the multivariate analysis was low PIWIL4 expression (HR = 2.185; 95% CI: 1.313–3.636; $p = 0.003$) (Table S3). Thus, these results highlight the detrimental role of low expression of PIWIL4 and allow the identification of two different risk subgroups of PC patients to be managed with differential treatment strategies to improve survival.

Table 3. Uni- and multivariate proportional hazards model for progression-free and overall survival of patients from the validation cohort.

	Univariate PFS (95% CI)				**Univariate OS (95% CI)**			
	HR	**Lower**	**Upper**	***p***	**HR**	**Lower**	**Upper**	***p***
Age (< 65 years vs. > 65 years)	1.060	0.604	1.860	0.840	1.198	0.723	1.986	0.484
Gender (Male vs. Female)	1.494	0.920	2.425	0.104	1.182	0.785	1.778	0.423
Diabetes Mellitus (No vs. Yes)	1.070	0.614	1.864	0.811	1.113	0.694	1.784	0.658
Adjuvant treatment (Yes vs. No)	1.016	0.556	1.857	0.959	1.226	0.781	2.094	0.456
Size (<2 cm vs. >2 cm)	3.023	1.413	6.465	0.004	1.255	0.754	2.087	0.382
pT (I / II vs. III)	1.682	1.033	2.738	0.037	1.679	1.110	2.540	0.014
Stage (I vs. II)	1.866	1.105	3.151	0.020	1.795	1.148	2.807	0.010
Grade (low vs. high)	1.406	0.695	2.845	0.343	1.221	0.664	2.245	0.522
Lymph nodes involved (No vs. Yes)	1.548	0.943	2.540	0.084	1.573	1.025	2.414	0.038
Vascular invasion (No vs. Yes)	1.348	0.807	2.252	0.254	1.481	0.959	2.287	0.077
Neural invasion (No vs. Yes)	1.757	1.027	3.007	0.040	1.658	1.060	2.593	0.027
PIWIL3 (high vs. low)	1.380	0.819	2.327	0.227	1.237	0.798	1.917	0.342
PIWIL4 (high vs. low)	1.979	1.178	3.325	0.010	2.093	1.344	3.260	0.001
	Multivariate PFS (95% CI)				**Multivariate OS (95% CI)**			
Size (<2 cm vs. > 2 cm)	3.095	1.237	7.744	0.016				
pT (I / II vs. III)	1.339	0.609	2.944	0.467	1.178	0.608	2.284	0.627
Stage (I vs. II)	1.596	0.655	3.890	0.304	1.691	0.683	4.188	0.256
Lymph nodes involved (No vs. Yes)					1.084	0.549	2.141	0.817
Neural invasion	1.232	0.620	2.449	0.551	1.229	0.761	1.985	0.398
PIWIL4 (high vs. low)	2.036	1.025	4.044	0.042	2.185	1.313	3.636	0.003

PFS: progression-free survival; OS: Overall survival; HR: hazard ratio; CI: confidence interval; vs.: versus; cm: centimeters.

In view of these results, we verified whether PIWIL3 or PIWIL4 could be related to any of the pathological characteristics registered in our study (Table S4). In this analysis, low levels of

PIWIL3 associated significantly with neural invasion ($p = 0.050$). Low PIWIL4 expression associated significantly with female patients ($p = 0.050$). Furthermore, a higher percentage of patients with T3 tumors associated significantly with low PIWIL4 expression ($p = 0.020$); the same occurred with neural invasion and low PIWIL4 expression ($p = 0.019$) (Table 4). These results suggest the lack of PIWIL4 expression as a deleterious effect in PC and support previous survival results.

Table 4. Statistical association between PIWIL3 and PIWIL4 protein expression with clinico-pathological characteristics.

	PIWIL3 Low	**PIWIL3 High**		**PIWIL4 Low**	**PIWIL4 High**	
Parameters	***N* (%)**	***N* (%)**	***p*-Value**	***N* (%)**	***N* (%)**	***p*-Value**
Gender			0.946			0.050
Male	43 (34%)	20 (16%)		34 (26%)	29 (23%)	
Female	44 (34%)	21 (16%)		46 (36%)	19 (15%)	
Age			0.630			0.227
<65 years	18 (14%)	7 (5%)		13 (10%)	12 (9%)	
>65 years	69 (54%)	34 (27%)		67 (53%)	36 (28%)	
Diabetes Mellitus			0.724			0.939
No	59 (49%)	29 (24%)		54 (45%)	34 (28%)	
Yes	21 (17%)	12 (10%)		20 (16%)	13 (11%)	
Stage			0.791			0.204
I	30 (25%)	16 (13%)		26 (22%)	20 (16%)	
II	50 (42%)	24 (20%)		49 (41%)	25 (21%)	
pT			0.503			0.020
I/II	48 (38%)	26 (21%)		40 (32%)	34 (27%)	
III	36 (29%)	15 (12%)		38 (31%)	13 (10%)	
Adjuvant treatment			0.704			0.085
No	50 (51%)	25 (25%)		52 (53%)	23 (23%)	
Yes	17 (17%)	7 (7%)		12 (12%)	12 (12%)	
Size			0.264			0.705
<2 cm	19 (19%)	12 (12%)		19 (19%)	12 (12%)	
>2 cm	50 (50%)	19 (19%)		45 (45%)	24 (24%)	
Lymph nodes involved			0.956			0.713
No	47 (39%)	23 (19%)		43 (36%)	27 (22%)	
Yes	34 (28%)	17 (14%)		33 (27%)	18 (15%)	
Vascular Invasion			0.950			0.875
No	51 (43%)	24 (20%)		46 (34%)	29 (30%)	
Yes	29 (25%)	14 (12%)		27 (25%)	16 (11%)	
Neural Invasion			0.050			0.019
No	27 (23%)	20 (17%)		23 (20%)	24 (20%)	
Yes	53 (45%)	18 (15%)		50 (42%)	21 (18%)	
Grade			0.095			0.917
Low	68 (55%)	37 (30%)		65 (52%)	40 (32%)	
High	16 (13%)	3 (2%)		12 (10%)	7 (6%)	

N: Number of patients; cm: centimeters.

4. Discussion

PC is an extremely lethal malignancy, in which an early diagnosis is crucial to increase patient survival. Therefore, molecular biomarkers will play an important role in the future management of this neoplasm. To date, the only biomarkers approved by the Food and Drug Administration (FDA) for PC are preoperative levels of CA19-9; however, the applicability of this biomarker has been questioned due to the fact that the biliary obstruction can also increase CA19-9 levels, not to mention that up to 10% of the population cannot synthesize this antigen [47]. Therefore, new biomarkers that combine high

sensitivity and specificity are needed in the clinical management of PC. Recently, novel proteins called PIWI have been discovered, and their expression was found in several types of tumors; thus, these factors may provide new perspectives in the clinical practice of PC [12]. In the present study, we have evaluated the expression of the four members of the PIWI family in PC-derived cell lines and one normal pancreatic cell line used as control. Interestingly, both PIWIL1 and PIWIL2 presented nearly undetectable expression levels in all cell lines. Indeed, this fact could be explained by the presence of CpG islands in the promoter region of *PIWIL1* [48] and *PIWIL2* [49]. It has been reported that downregulation of PIWIL1 and PIWIL2 by promoter CpG island hypermethylation has been observed in other types of tumors like testicular or non-small cell lung cancer [38]. It has also been described how PIWIL1 downregulation regulates migration of Schwann cells for peripheral nerve regeneration after injury [50]. Since we found low levels of PIWIL1 and PIWIL2 in a pancreatic normal cell line, this event seems not to be exclusive of tumor cells. In fact, these genes play crucial roles in spermatogenesis, and their downregulation impairs germ cell development that might associate with male infertility [51].

On the other hand, PIWIL3 and PIWIL4 showed higher protein levels and a differential expression pattern throughout cell lines, which includes a non-tumor cell line. This first attempt implied that PIWIL3 and PIWIL4 might not act as an oncogene in PC. Nevertheless, the role of PIWIL3 and PIWIL4 in tumorigenesis is rather controversial. For this, we decided to evaluate their role with functional experiments in tumor cell lines and a non-tumor cell line as normal control. Some studies have reported the expression of these proteins with oncogenic features; e.g., one study described how cancer cells re-express PIWIL3 to promote cancer cell growth [52]. Other research highlighted that PIWIL3 and PIWIL4 presented oncogenic potential in several types of cancers [13]. In contrast, PIWIL3 exhibited a protective effect in glioma cells [25], and low expression of PIWIL4 has been found in tumor cells from hepatocellular carcinoma [36], breast cancer [22] and non-small cell lung cancer [38]. Therefore, the role of PIWIL3 and PIWIL4 in tumor initiation and development remains still unclear. In our functional experiments, we were able to evaluate cell response to PIWIL3 and/or PIWIL4 downregulation. Moreover, the inclusion of a non-tumor cell line in these experiments led us to discern between a true oncogenic role and a normal cell function. Our experiments, designed to evaluate cell motility, chemoresistance and undifferentiated phenotype, revealed that the effect observed after PIWIL3 and/or PIWIL4 downregulation in tumor cells were also shown by the non-tumor cell line. Here, we observed how PIWIL3 and PIWIL4 knockdown decreased motility of both tumor and normal cells through a mesenchymal arrest in favor of the epithelial phenotype. This reduction of the cell motility by PIWIL4 downregulation has previously been described in breast cancer cells through an impairment of Vimentin and N-Cadherin [33]. However, this study only provided evidence of migration delay in MCF-7 tumor cell line but not in a non-tumor cell line. Then, it is still unknown whether PIWIL4 downregulation exclusively affects cell motility of breast cancer cells or also impairs motility of normal cells. For this concern, it has been reported how PIWIL2 regulates invasion abilities of prostate cancer cells through modulation of EMT protein expression [53]. HPV16 is also able to increase PIWIL2 levels to increase proliferation and invasion of cervical cancer cells [54]. However, not only do PIWI proteins play a role as invasion promoting factors, but also their associated piRNAs. It has been described how downregulation of piRNA-36712 promotes invasion and migration of tumor cells; thus, it is considered a potential tumor suppressor in breast cancer [55]. Another study supports the tumor-suppressive properties of piR-823 because its upregulation inhibits tumor cell growth in gastric cancer models [56]. In addition, piR-823 downregulation suppressed cell proliferation of colorectal cancer cells by a direct modulation of the transcriptional activity of HSF1 [57]. Other functional experiments have demonstrated that piR-651 promotes tumor formation in non-small cell lung cancer mediated by Cyclin D1 and CDK4 [58]. To the best of our knowledge, we have described for the first time the implication of PIWIL3 and PIWIL4 in cell motility through EMT modulation of tumor and non-tumor pancreatic cells. From a clinical point of view, this connection between PIWIL3/PIWIL4 and EMT should be managed carefully since EMT is the most critical mechanism by which adult tissues, including pancreatic β-cells, are repaired after inflammatory, toxic or trauma injuries [59–61].

Many works have reported that PIWI proteins have the ability to regulate transposable elements to maintain genomic stability of stem cells [62]. In our functional studies, we observed a diminished undifferentiated phenotype of pancreatic cells, and we found a decrease in the number and size of pancreatic stem-cell-like spheres after PIWIL3 and/or PIWIL4 downregulation. This result supports the role of PIWIL3/PIWIL4 in the maintenance of undifferentiated phenotype both in tumor and in normal cells, as was previously observed in normal spermatogenesis of mammals [63]. Moreover, downregulation of PIWIL2 decreased proliferation and survival of breast cancer stem cells through a decrease in the protein levels of STAT3, BCL-XL and Cyclin D1 [64]. This link between PIWI proteins and undifferentiated phenotype has also been demonstrated when downregulation of PIWI proteins impaired whole-body regeneration of certain marine organisms [65]. Hence, the role of PIWIL3/PIWIL4 seems not to be exclusive of tumorigenesis and suggests a crucial function in fundamental tissue maintenance.

Since expression of PIWI proteins increased resistance to drugs in cervical cancer [66] and in non-small cell lung cancer [67], we decided to evaluate whether PIWIL3 or PIWIL4 were able to modulate chemoresistance of PC. Here, we described how PIWIL3 and PIWIL4 downregulation increases the effect of the gold standard chemotherapies against PC. Surprisingly, PL45 cell line showed no effect after individual or combined downregulation. However, the lack of effect in PL45 could be explained not only by its mutations in *KRAS*, *TP53* or *DPC4*, which are commonly found in PC, but also by its mutation in *BRCA2* gene, which could confer chemoresistance in PC as recently described by Wang et al. [68]. As we observed and as previously reported in the literature, non-tumor cell line hTERT-HPNE showed Gemcitabine resistance [69]. Nevertheless, it reverted completely its chemoresistance after PIWIL3 and/or PIWIL4 knockdown and significantly increased the effect of Gemcitabine alone or in combination with Nab-Paclitaxel. However, this statistically significant drug response exhibited after double downregulation achieved neither additive nor synergic effect compared with individual protein downregulation in the presence of single treatment or combination. The fact that PIWIL3 and/or PIWIL4 downregulation increased considerably drug response on the normal cell line does not make modulation of PIWIL3 or PIWIL4 suitable for future drug design against PC. This effect on normal cells could imply higher toxicity and adverse events, which could compromise tolerability and safety of patients. In order to explain the link between these two PIWI proteins and chemoresistance, we explored factors related to Gemcitabine or Nab-Paclitaxel resistance in PC. Hepatocyte Nuclear Factor Alpha (HNF4A) appeared rapidly as a potential factor that may account for this finding. HNF4A is overexpressed in hepatocytes, enterocytes and pancreatic β-cells. It also ensures expression of intermediary genes required for metabolism of glucose and lipids, and it is necessary for cell differentiation [70]. In PC, high expression levels of HNF4A have been correlated with poor prognosis. HNF4A has been described as conferring chemoresistance in other types of tumors like breast cancer, where it has been the most upregulated gene after hypoxic conditions and led to a higher Doxorubicin resistance [71]. Indeed, a synthetic HNF4A antagonist is under investigation to selectively eradicate cancer cells [72]. Moreover, the mechanism of HNF4A to confer chemoresistance to Gemcitabine is through (a) direct regulation of hENT1, which is responsible for Gemcitabine uptake of tumor cells [46]. At first glance, neither PIWIL3 nor PIWIL4 exhibited a correlation with hENT1. Nevertheless, a high trend towards significance was found between PIWIL3 and HNF4A at the protein level, and a statistically significant correlation was found between PIWIL4 and HNF4A both at mRNA and at the protein level. Therefore, these results support the role of these PIWI proteins as crucial factors for regulation of chemotherapy uptake of cells.

Finally, we assessed survival analyses by staining PIWIL3 or PIWIL4 in PC samples. We were struck in particular by the fact that PIWIL3 and PIWIL4 were expressed in pancreatic normal tissues [73,74]; consequently, our hypothesis as oncogenes was found baseless and was simply discarded. Furthermore, survival analyses revealed that low expression of PIWIL4 associated significantly with both shorter progression-free and overall survival. These results suggested a deleterious effect of low levels of PIWIL4. Since PC is a deadly disease and survival of patients is rather limited, our findings allow the identification of two different risk subgroups of PC patients that can be clinically managed

independently to improve survival. Only tumor size higher than 2 cm emerged as statistically significant together with low PIWIL4 expression in Cox multivariate analysis for progression-free survival. This result could be expected, since tumor size at diagnostic is closely related to survival. It has been reported that the 5-year survival rate is around 50% when tumors are below 2 cm [75] and close to 100% when tumors are below 1 cm [76]. Moreover, we found that a higher percentage of patients with low PIWIL4 expression exhibited a link with T3 tumors and neural invasion compared with those with high PIWIL4 expression.

On the other hand, the fact that low levels of PIWIL4 are related to reduced cell motility seemed to go against our results that suggest it as a poor prognostic biomarker of PC. However, our results suggest that the lack of PIWIL4 could increase treatment toxicity and adverse events to patients, an impaired tissue repair driven by a delay in cell motility through EMT reversion, and a default on cell differentiation. All these mechanisms could retard the healing process of PC patients and lead to shorter progression-free and overall survival.

5. Conclusions

In our study, we have compiled some functional experiments and survival analysis according to PIWIL3 or PIWIL4 expression to dissect the role of these proteins in PC. Our findings support PIWIL3 and PIWIL4 as crucial factors in the regulation of cell motility, stem cell maintenance and drug resistance both in tumor and healthy pancreatic cells. Moreover, low PIWIL4 expression is able to predict shorter survival of PC patients. These results provide new insights into the knowledge of PIWI proteins functions and their controversial role in tumorigenesis.

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Author Contributions: J.M.-U. and J.G.-F. designed the study. L.O.-M., S.G.-B., E.P.-A., L.D.-V. and A.C. collected clinical samples and elaborated the database. W.L. and N.G.-C. performed the experiments. J.M.-U., W.L., A.O. and N.G.-C. analyzed the data of the in vitro experiments. M.J.F.-A. and L.O.-M. evaluated and scored immunohistochemical stainings. J.M.-U. wrote the paper. J.M.-U., W.L., A.O., and N.G.-C. critically revised the manuscript. J.G.-F. provided funding. All authors have read and agreed to the published version of the manuscript.

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Supplementary Materials: Materials and Methods

Cell Lines and Cell Culture

The human PC-derived cell lines PANC 04.03 (CRL-2555), PL45 (CRL-2558), BxPC-3 (CRL-1687) and one non-tumor human pancreatic ductal epithelial cell line hTERT-HPNE (CRL-4023) were purchased and cultured under American Type Culture Collection (ATCC) recommendations. RWP1 and PANC-1 were kindly provided by Dr. Fatima Gebauer (CRG, Barcelona, Spain). RWP1, PANC-1 cells were routinely grown in RPMI supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (P/S). All cell lines were maintained at 37 °C in a humidified atmosphere with 5% CO2.

Patient Samples

A total of 44 pancreatic cancer patients from Hospital Fundacion Jimenez Diaz and 182 pancreatic cancer patients who underwent surgery from 2006 to 2012 were assessed for eligibility. Patients were followed-up until March 2019. Tumors were surgically resected and formalin-fixed and paraffin-embedded (FFPE) immediately for pathologic diagnosis. Tissue microarrays (TMA) were constructed with available FFPE tumor samples. All patients that presented positive margins of resection (R1) were excluded from the study. To assess survival analysis, only patients with available data of progression-free or overall survival were included in the study. Two experienced pathologists reviewed tumor histology (M.J.F.-A. and L.O.-M.).

Ethics Statement

All human samples were kindly supplied by the Biobank of Fundacion Jimenez Diaz Hospital (PT13/0010/0012) and by the BioBank of University Hospital Clinico San Carlos (B.0000725; PT17/0015/0040; ISCIII-FEDER). The institutional review board (IRB) of the University Hospital Clinico San Carlos evaluated the present study, granting approval on Mars 10th, 2017 with approval number nº 17/091-E. The institutional review board (IRB) of University Hospital Fundacion Jimenez Diaz approved the study 15 November 2016 under the approval number 19/16. All patients gave written informed consent for the use of their biological samples for research purposes. Moreover, fundamental ethical principles promoted by Spain (LOPD 15/1999) and the European Union Fundamental Rights of the EU (2000/C364/01) were followed. In addition, all patient's data were processed according to the Declaration of Helsinki (last revision 2013) and Spanish National Biomedical Research Law (14/2007, of 3 July).

Western Blot

Total protein from PC-derived cell lines and controls were extracted with RIPA buffer supplemented with protease inhibitor cocktail (Roche). Samples were fractionated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (BioRad). Membranes were incubated overnight at 4 °C with the following primary antibodies: PIWIL1(1:500; ab12337; Abcam), PIWIL2 (1:1000; ab181340; Abcam), PIWIL3 (1:100; sc-398779; Santa Cruz Biotechnology), PIWIL4 (1:500; ab111714; Abcam) and Actin (1:10000; a1978; Sigma-Aldrich). To quantify the expression of EMT markers after PIWIL3 or PIWIL4 downregulation, membranes were incubated with the following primary: Fibronectin (1:1000; ab2413; Abcam), Vimentin (1:1000; 5741s; Cell Signaling), E-Cadherin (1:1000; 3195s; Cell Signaling), Occludin (1:1000; sab4200593; Sigma-Aldrich) and Slug (1:1000; ab27568; Abcam). We used an anti-rabbit (NA934V; GE Healthcare) as a secondary antibody for PIWIL1, PIWIL2, PIWIL4, Fibronectin, Vimentin, E-Cadherin, Occludin and Slug, and an anti-mouse secondary antibody (NA931V; GE Healthcare) for PIWIL3 and Actin—both secondary antibodies were conjugated with horseradish peroxidase. For band densitometry we used

the software ImageJ version 1.50i (National Institutes of Health, USA). Protein extracted from mouse testis was used as controls.

RNA Interference

For PIWIL3 and/or PIWIL4 knockdown, we used two independent silencing sequences individually (Thermo Fisher Scientific). All cell lines were firstly transfected at 60%–70% confluence using X-tremeGENE Transfection Reagent (Roche) according to manufacturer's instructions, and a second transfection was carried out in tumor cell lines after 48 h to maximize downregulation. Since PL45 expressed the highest levels of PIWIL3, we have to use a combination of two inhibitory sequences against PIWIL3. Table S1 shows each individual or combination of inhibitory sequences for each cell line according to PIWIL3, PIWIL4 or PIWIL3 and PIWIL4 downregulation. As a control, each cell line was transfected with a scrambled siRNA (sc-37007, Santa Cruz). All subsequent experimental procedures were evaluated at the same day of maximum protein downregulation.

Table S1. Different inhibitory sequences used individually or in combination to downregulate PIWIL3, PIWIL4 or PIWIL3 and PIWIL4.

Cell Line	PIWIL3 Downregulation	PIWIL4 Downregulation	PIWIL3 and PIWIL4 Downregulation
RWP1	s54203 (si03) or s54205 (si05)	s44572 (si72) or s44573 (si73)	s54203 (si03) + s44572 (si72) or s54205 (si05) + s44573 (si73)
PL45	s54203 (si03) + s54204 (si04) or s54204 (si04) + s54205 (si05)	s44571 (si71) or s44573 (si73)	s54203 (si03) + s54204 (si04) + s44571 (si71) or s54204 (si04) + s54205 (si05) + s44573 (si73)
hTERT-HPNE	s54204 (si04) or s54205 (si05)	s44571 (si71) or s44573 (si73)	s54204 (si04) + s44571 (si71) or s54205 (si05) + s44573 (si73)

Wound healing and Boyden Chamber Migration Assay

Cell motility after PIWIL3 and/or PIWIL4 downregulation was estimated by wound healing assays. Cells were grown as a monolayer, and an artificial homogenous wound was created with a sterile plastic 10 μL micropipette tip. The growth of cells in the wound was measured at 0, 6, 12 and 24 h. Boyden chamber migration assays were performed in cell culture inserts with 8-μm pores in 24-well plates (Corning). Cell lines were seeded at a density of 5×10^4 cells per insert in 150 μL growth medium without FBS. The recipient wells received 600 μL growth medium supplemented with 20% FBS. The migration was determined after 24 h. Afterwards, cells were fixed and stained with toluidine blue (Sigma-Aldrich). The non-migrated cells on the upper side of the membrane were removed with a cotton swab. Membranes were cut and fixed in microscope slides, and photographs were taken with a stereo microscope (Leica DMi1). Three independent experiments were done, and all experiments were performed in triplicate wells.

Cytotoxicity Assay

Tumor cell lines were treated for 48h with previously determined IC_{50} of Gemcitabine (RWP1:6 nM, PL45: 358nM), Nab-Paclitaxel (RWP1: 11 μM, PL45: 143 μM). Since hTERT-HPNE presented resistance to Gemcitabine, a concentration of 250 μM was used. hTERT-HPNE were also cultured in IC_{50} of Nab-Paclitaxel (236 μM). To determine doses for the combination of Nab-Paclitaxel plus Gemcitabine, IC_{25} dose of Nab-Paclitaxel was set for each cell line due to its high toxicity; then, different concentrations of Gemcitabine were tested to achieve 50% of cell death according to Awasthi N. et al. (42). Therefore, treatment combination for RWP1: 0.36nM of Gemcitabine + 3 μM of Nab-Paclitaxel; for PL45: 156nM of Gemcitabine + 41 μM of Nab-Paclitaxel; for hTERT-HPNE: 14 μM of Gemcitabine + 90 μM of Nab-Paclitaxel. Drugs were kindly provided from the Pharmacology Department of Fundacion Jimenez Diaz Hospital. Cell viability was determined by absorbance with 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction assay (Promega). We performed three replicates of each experiment in triplicate.

Tumor Sphere Formation and Flow Cytometry

PL45, RWP1 and hTERT-HPNE cell lines were separately seeded into ultra-low attachment 6-well plate with 1.5 mL sphere formation medium (SFM) at a concentration of 5000 or 10,000 cells/well, respectively. The SFM consisted of DMEM/F12 medium (11330-032, Gibco) supplemented with 20 ng/mL Epidermal Growth Factor (EGF, 236-EG-200, R&D Systems), 20 ng/mL basic Fibroblast Growth Factor (bFGF, 233-FB-025, R&D Systems), 2% B27 supplement (17504044, Gibco), 1% N2 supplement (17502048, Gibco) and 1% Penicillin-Streptomycin (P/S). Subsequently, cells were cultured at 37 °C in a 5% CO2 humidified environment to form spheroid structures. Photographs were taken with a stereomicroscope (Leica DMi1). Dedifferentiation was evaluated by cytometry to detect CD24+/CD133+/EPCAM+ cells. For this, tumor spheres were dissociated with trysin-EDTA and incubated in presence of the following antibodies: CD24-APC (17-0242-82; BD Bioscience), CD133-FITC(11-1339-42; BD Bioscience) and EPCAM-PE (12-5791-82; BD Bioscience). Cells were then acquired and analyzed on a flow cytometer (FACS Aria II; Becton Dickinson). Three independent experiments were done and all experiments were performed in triplicate.

Immunohistochemistry

Tissue microarrays with 182 patient samples were constructed for immunohistochemistry analysis and contained 364 cores (2 cores per patient) using the MTA-1 tissue arrayer (Beecher Instruments, Sun Prairie, USA). Each core (diameter, 1 mm) was punched from pre-selected tumor regions in paraffin-embedded tissues. Staining was conducted in 2-µm sections. Slides were deparaffinised by incubation at 60 °C for 10 min and incubated with PT-Link (Dako, Denmark) for 20 min at 95 °C in a low pH buffered solution. To block endogenous peroxidase, holders were incubated with peroxidase blocking reagent (Dako, Denmark). Biopsies were incubated for 20 min with a 1:100 dilution of anti-PIWIL1 antibody (ab12337; Abcam), 1:250 dilution of anti-PIWIL2 antibody (ab181340; Abcam), 1:100 dilution of anti-PIWIL3 antibody (ab77088; Abcam), 1:25 dilution of anti-PIWIL4 antibody (ab111714; Abcam) or 1:100 dilution of anti-HNF4A antibody (ab92378; Abcam Cambridge, UK). Tissues were incubated with the appropriate anti-Ig horseradish peroxidase-conjugated polymer (EnVision, Dako, Denmark) to detect antigen–antibody reaction. All antibodies and anti-Ig horseradish peroxidase-conjugated antibody presented high specificity, and no positiveness resulted from these antibodies individually. To determine the best immunohistochemistry conditions, human testis tissues were used as a positive control for PIWIL1, PIWIL2, PIWIL3 and PIWIL4 antibodies and human colon tissues for HNF4A antibody according to The Human Protein Atlas (http://www.proteinatlas.org). Sections were then visualized with 3,3′-diaminobenzidine as a chromogen for 5 min and counterstained with hematoxylin. Photographs were taken with a stereomicroscope (Leica DMi1). To quantify the PIWIL3 and PIWIL4 immunostaining, a semiquantitative HistoScore (Hscore) was calculated. The Hscore was determined by estimation of the percentage of positively stained cells with low, medium or high intensity of staining, after applying a weighting factor following the formula Hscore = (low %) × 1 + (medium %) × 2 + (high %) × 3, and the results ranged from 0–300. To identify the best cut-off point to separate patients according to PIWIL3 or PIWIL4 protein expression and the risk of progression and death on disease, we performed ROC curves. However, the cut-off point according to ROC curves did not separate patients' survival. Therefore, we stratified patients into tertiles according to their PIWIL3 or PIWIL4 Hscore, and the third tertile was considered high PIWIL3 or PIWIL4 expression. HNF4A immunostaining was categorized as positive or negative since HNF4A exhibited a clear nuclear positiveness. Quantification for each patient biopsy was calculated with the average of both cores by two independent researchers.

Statistical Analysis

In wound healing assays, distances between gaps have been measured and a U Mann–Whitney test evaluated differences in length compared to control scramble at 24 h. For evaluation of Boyden chamber assay, cells from each condition from 10 randomly selected fields (10X objective) were

counted and each condition was compared to control scramble with U Mann–Whitney test. Those tumor spheres higher than 70 μm were counted and their sizes were determined by image processing. Statistical analyses between each downregulation and control scramble were assessed with a U Mann–Whitney test. In the cytotoxicity assay, absorbencies were normalized with the absorbance of untreated cells and IC_{50} for each cell line, and treatment was assessed by curve-fitting through nonlinear regression using the Solver tool of Microsoft Excel software. We calculated the drug effect by subtracting absorbance of untreated cells from that of treated conditions. We analyzed differences between each condition and control scramble with non-parametric U Mann-Whitney test. Statistical correlation between hnf4a and hent1 with piwil3 or piwil4 at mRNA level was assessed with Pearson since all variables were normally distributed. Linear correlation was evaluated and interpreted by Pearson's r. Association between HNF4A and PIWIL4 at the protein level was analyzed with Chi-square test. Progression-free survival (PFS) and overall survival (OS) curves according to PIWIL3 or PIWIL4 at mRNA or protein level were performed with Kaplan-Meier, and survival was analyzed with log-rank test. *p*-values ≤ 0.05 were considered statistically significant. All statistics were performed with the IBM SPSS statistics 20.0.

ARTÍCULO 3: UNR/*CDSE1* expression as prognosis biomarker in resectable pancreatic ductal adenocarcinoma patients: A proof-of-concept

El gen *CDSE1* se encuentra próximo al extremo 5´-UTR de *NRAS* y codifica una proteína de unión a ARN denominada UNR. El objetivo de este estudio fue analizar la expresión de UNR y su correlación con el resultado en pacientes con adenocarcinoma ductal pancreático resecable.

Para esto, hemos utilizado muestras de pacientes con CaPa resecables que se sometieron a duodenopancreatectomía para evaluar la expresión de la proteína UNR por inmunohistoquímica utilizando un microarray de tejidos. Aquí, observamos que la baja expresión de UNR se asoció significativamente con una supervivencia libre de progresión más corta después de la cirugía (P=0.010). Además, este marcador pronóstico se mantuvo significativo después del modelo de riesgos proporcionales de Cox (P=0.036). Además, estudiamos el papel de la expresión de csde1 a nivel de ARNm en el pronóstico de pacientes utilizando datos de repositorios públicos (GEO y TGCA), confirmando nuestros resultados. Curiosamente, la expresión de csde1 se correlacionó con la de los genes característicos de un subtipo molecular inmunogénico de cáncer pancreático.

Aportación Personal al trabajo:

En este trabajo mi aportación se centró en evaluar la expresión de la proteína UNR por inmunohistoquímica en pacientes con CaPa resecable. Después realicé un análisis estadístico de los resultados, y me encargué de la búsqueda y análisis de datos de repositorios públicos (GEO y TGCA) para confirmar nuestros resultados. También ayudé a analizar la expresión de csde1 a nivel de ARNm con los genes característicos de otros subtipos moleculares de CaPa.

PLOS ONE

RESEARCH ARTICLE

UNR/*CDSE1* expression as prognosis biomarker in resectable pancreatic ductal adenocarcinoma patients: A proof-of-concept

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Abstract

Pancreatic ductal adenocarcinoma is an aggressive form of pancreatic cancer and the fourth leading cause of cancer-related death. When possible, curative approaches are based on surgical resection, though not every patient is a candidate for surgery. There are clinical guidelines for the management of these patients that offer different treatment options depending on the clinical and pathologic characteristics. However, the survival rates seen in this kind of patients are still low. The *CDSE1* gene is located upstream of *NRAS* and encodes an RNA-binding protein termed UNR. The aim of this study was to analyze UNR expression and its correlation with outcome in patients with resectable pancreatic ductal adenocarcinoma (PDAC). For this, samples from resectable PDAC patients who underwent duodenopancreatectomy were used to evaluate UNR protein expression by immunohistochemistry using a tissue microarray. Here, we observed that low UNR expression was significantly associated with shorter progression-free survival after surgery ($P = 0.010$). Moreover, this prognostic marker remained significant after Cox proportional hazards model ($P = 0.036$). We further studied the role of *CDSE1* expression in patient's prognosis using data from public repositories (GEO and TGCA), confirming our results. Interestingly, *CDSE1* expression correlated with that of genes characteristic of an immunogenic molecular subtype of pancreatic cancer. Based on these findings, UNR may be considered a potential prognostic biomarker for resectable PDAC and may serve to guide subsequent adjuvant treatment decisions.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) has higher incidence in industrialised countries [1] and is the fourth leading cause of cancer death in both sexes in the USA, where 53,070 new cases of PDAC were diagnosed in 2016 [2]. Moreover, it is the eighth leading cause of cancer death in men and the nineth in women worldwide [3]. It has been reported that the 5-year survival rate is 50% when tumors are < 2 cm in size [4] and close to 100% for tumors < 1 cm [5]. Although these data are encouraging, PDAC is usually asymptomatic, and the disease only becomes apparent after the tumor invades surrounding tissues or metastasizes to distant organs [6]. In fact, distant metastasis is found in 53% of PDAC patients at the time of diagnosis [2]. To date, surgical resection remains the best management option for PDAC originating in the ampulla of Vater, bile duct, or pancreas. Patient's prognosis has been predicted based on pathological characteristics such as tumor size, grade of differentiation, lymph-node status, etc [7]. Several prognostic biomarkers have been suggested, such as Smad4 or MUC1; also, predictive biomarkers including SPARC, HuR, or members of the BRCA2 family have been described [8–11]. To date, preoperative levels of carbohydrate antigen 19–9 (CA 19–9) are the only prognostic biomarker approved by the Food and Drug Administration (FDA) for use in cases of resectable PDAC [12]. This marker shows a relatively high sensitivity and specificity for PDAC [13], providing results that are superior to those of other markers, such as carcinoembryonic antigen (CEA), carbohydrate antigen 50 (CA-50), and DUPAN-2 [14, 15]. However, the applicability of CA 19–9 is compromised by the fact that biliary obstruction can increase its serum levels [16], and up to 10% of the population cannot synthesise this antigen [17].

In the late 1980s, an active transcription unit called UNR (Upstream of N-ras) was discovered and subsequently included in the RNA-binding protein (RBP) family due to its ability to bind single-stranded RNA [18]. RBPs are pivotal components in the determination of messenger RNA (mRNA) and microRNA function, as they control transcript biogenesis, localization, degradation, and activity. Alteration of RBP function can lead to impairment of any of the crucial steps of RNA processing, and deregulation of RBP expression or activity has been reported in several malignancies [19]. Moreover, several RBPs have been shown to play a key role in cancer via regulation of mRNA splicing, translation, and stability [20]. *In vitro* assays indicated that UNR could interact with cytoplasmic RNA in a sequence-specific manner [18, 21]. Subsequent studies demonstrated that UNR acts as an RNA chaperone by changing the structure of the IRES into one that is functionally competent for translation [22]. Other reports showed that UNR compensates X-chromosome dosage in Drosophila [23] and prevents differentiation of embryonic stem cells in mouse models [24].

In the cancer context, UNR has been shown to regulate proto-oncogenes like c-fos [25] and c-myc [26]. In addition, UNR promotes melanoma progression by regulating the expression of Pten, Rac1 and Vimentin, among other genes [27]. Interestingly, overexpression of *HEPSIN*, one of the most consistently up-regulated genes in prostate-cancer patients [28], inhibits the expression and IRES activity of UNR in cancer-derived cell lines [29]. In contrast, knockdown of *HEPSIN* expression with siRNA led to an increase of UNR and up-regulation of its IRES activity [29]. Curiously, UNR is transcribed from the same strand of DNA as the *NRAS* proto-oncogene [30], and its expression has been reported to down-modulate *NRAS* expression through mRNA accumulation in tissues [31]. Altogether, these data point to diverse roles of UNR in cancer development.

The role of UNR in PDAC has not been previously addressed. In this study, we aimed to quantify UNR protein expression and evaluate its role as a potential marker to determine

outcome of PDAC patients. We have further analysed the association between UNR/*CDSE1* expression and different molecular subtypes of pancreatic cancer.

Materials and methods

Patient samples

A total of 53 patients with pancreatic adenocarcinoma who underwent pancreaticoduonenectomy from 2007 to 2013 at the Hepatobiliary and Pancreatic Surgery Unit (General and Digestive Tract Surgery Department, Fundación Jiménez Díaz University Hospital) were assessed for eligibility. All cephalic duodenopancreatectomy specimens have been sectioned and embedded *in toto* following Verbeke *et al.* scheme [32]. This scheme allows accurate establishment of the origin of the tumor in the pancreas, the extrahepatic biliary tract or the duodenum. Twenty-two patients were excluded due to insufficient sample quality for immunohistochemistry, patients lost to follow-up, or tumors having duodenal origin. Most of the tumors studied were in stage II (78%). Gemcitabine was administered alone or in combination with radiotherapy as adjuvant treatment post-surgery in one-third of the cases included (32%). All tumor samples included in this study were confirmed to be low-grade resectable pancreatic adenocarcinomas based on the recommendations of the College of American Pathologists [33].

Immunohistochemistry and quantification

A tissue microarray was constructed for immunohistochemistry analysis and contained 62 cores (2 cores per patient) using the MTA-1 tissue arrayer (Beecher Instruments, Sun Prairie, USA). Each core (diameter, 1 mm) was punched from pre-selected tumor regions in paraffin-embedded tissues. Staining was conducted in 2-μm sections. Slides were deparaffinised by incubation at 60˚C for 10 min and incubated with PT-Link (Dako, Denmark) for 20 min at 95˚C in a high pH buffered solution. To block endogenous peroxidase, holders were incubated with peroxidase blocking reagent (Dako, Denmark). Biopsies were incubated for 20 min with a 1:50 dilution of CDSE1 antibody (ab96124; Abcam, Cambridge, UK) and 1:1000 dilution of NRAS antibody (ab167136; Abcam, Cambridge, UK) followed by incubation with the appropriate anti-Ig horseradish peroxidase-conjugated polymer (EnVision, Dako, Denmark) to detect antigen-antibody reaction. Both CDSE1 antibody and anti-Ig horseradish peroxidase-conjugated antibody presented high specificity and no positiveness resulted from these antibodies individually. A human intestinal tissue was used as a positive control (according to the human protein atlas available at http://www.proteinatlas.org) for immunohistochemical staining and to determine CDSE1 antibody concentration. Sections were then visualised with 3,3'-diaminobenzidine as a chromogen for 5 min and counterstained with haematoxylin. Photographs were taken with a stereo microscope (Leica DMi1, Wetzlar, Germany). Immunoreactivity of tumor sample was quantified blind with UNR intensity of expression categorized as negative, low, medium or high expression according to Wurth *et al.* [27]. Quantification for each patient biopsy was calculated with the average of both cores by two independent pathologists.

Statistical analysis of immunohistochemical expression

The association between UNR expression and progression-free survival after resection was the primary endpoint, and overall survival was the secondary endpoint. Progression-free survival was defined as the interval between the dates of surgery and recurrence (local or distant). Overall survival was defined as the interval between the dates of surgery and death from any cause.

The association between UNR expression and clinico-pathological variables was evaluated by Fisher´s exact test.

The univariate Cox proportional hazards model was used to assess the hazard ratios and confidence intervals of both molecular and clinical variables.

TCGA-pancreatic cancer dataset analysis

Sixty patients from a group of 186 pancreatic cancer patients with RNA expression data in the TCGA database were eligible for overall survival analysis, while 47 patients were eligible for progression-free survival analysis (S1 Fig). We selected stages I/II low grade PDAC patients featuring histology with complete resections (R0) and follow-up, without *CDSE1* genetic alterations and untreated with neoadjuvant chemotherapy. For both progression-free and overall survival, ROC (Receiver Operating Characteristic) curves did not show a clear cut-off point (progression-free survival AUC = 0.578, $P = 0.129$; overall survival AUC = 0.583, $P = 0.065$; data not shown). Therefore, mean of Z-score was used as cut-off point for both survival analyses. Additionally, the TCGA dataset was analysed using cBioPortal [34, 35] to address gene expression and to calculate Pearson and Spearman correlation coefficients. Correlation coefficients were interpreted according to Cohen [36]. Values of 0.10 to 0.30 could be interpreted as a weak correlation, 0.30 to 0.50 as a moderate correlation and greater than 0.50 as a strong correlation [36]. Z-scores were plotted in a heatmap using Perseus_1.5.3.0.

GEO (GSE28735) dataset analysis

Survival analysis was assessed with the association between *CDSE1* Z-score and overall survival information of 42 pancreatic tumors that contained complete clinical follow-up from Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) dataset with accession number GSE28735 entitled: "Microarray gene-expression profiles of 45 matching pairs of pancreatic tumor and adjacent non-tumor tissues from 45 patients with pancreatic ductal adenocarcinoma". Expression profile of tumor samples were detected with Affymetrix GeneChip Human Gene 1.0 ST arrays. Z-score was stratified into tertiles (low $\leq$ 33%; 34% $<$ medium $\leq$ 66%; high $>$ 67%), and third tertile (high expression) was used as cut-off point.

Z-score for *CDSE1* mRNA expression was calculated as follows: Z-score = (log value of mRNA expression in tumor sample–log value of mRNA mean expression in reference samples) / log value of standard deviation of mRNA expression in reference samples. Reference samples have been considered the adjacent non-tumor tissues (for GSE28735 dataset) and all diploid tumors for the gene in question (for TCGA dataset). All survival curves were generated using the Kaplan-Meier method, and significant differences in survival between groups were determined by the log-rank test. P-values ≤ 0.05 were considered significant. Analysis was performed with the IBM SPSS programme, version 20.0.

Results

Patient characteristics

The clinical features of the PDAC patients included in the study are summarised in Table 1. Our cohort was well-balanced in terms of sex (48% males and 52% females). The median age of patients was 69 years (range 37–82 years). Pathologic diagnosis revealed the size of the resected tumors to be lower than 2 cm in 61% of cases. Twenty-two percent of tumors were stage I and 78% stage II. Negative surgical margins were found after surgery in 90% of cases. Fifty-eight percent of patients showed lymph-node involvement and most patients had neural and vascular invasion (74% and 71%, respectively). Adjuvant treatment based on gemcitabine alone or gemcitabine plus radiotherapy was administered post-surgery in 32% of patients

Table 1. Clinical characteristics of resectable low-grade pancreatic cancer patients.

Characteristics	N (%)
Age	
< 65 years	12 (39%)
> 65 years	19 (61%)
Sex	
Female	16 (52%)
Male	15 (48%)
Size	
< 2 cm	19 (61%)
> 2 cm	12 (39%)
Stage	
I	7 (23%)
II	24 (77%)
pT	
T1	5 (16%)
T2	3 (10%)
T3	23 (74%)
pN	
N0	12 (39%)
N1	18 (58%)
N/A	1 (3%)
Tumor location	
Pancreas	12 (39%)
Bile duct	10 (32%)
Ampulla	9 (29%)
Lymph nodes involved	
No	12 (39%)
Yes	18 (58%)
N/A	1 (3%)
Adjuvant treatment	
No	20 (65%)
Yes	10 (32%)
N/A	1 (3%)
Positive margins	
No	28 (90%)
Yes	3 (10%)
Vascular invasion	
No	9 (29%)
Yes	22 (71%)
Neural invasion	
No	8 (26%)
Yes	23 (74%)

N/A: not available

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based on the consensus of a multidisciplinary team. Gemcitabine was administered in 3–12 cycles depending on radiotherapy doses (45–54 Gy in 1.8–2.5 Gy fractions).

Low UNR expression level is associated with poor outcome in low-grade resected PDAC patients

To date, outcome of resected PDAC patients is clinically predicted according to pathologic criteria. For this reason, we first checked the statistical power of stage as a prognostic tool in our cohort of patients. For that purpose, the association between stage and survival of PDAC patients was assessed. However, Kaplan-Meier analysis revealed no statistically significant association between stage and progression-free survival ($P = 0.196$; data not shown) nor with overall survival ($P = 0.657$; data not shown).

Based on previous reports suggesting an association between RBPs and cancer, we hypothesised that UNR expression levels could be closely related to outcome in patients with PDAC. To test this hypothesis, a tissue microarray was constructed and stained to quantify UNR expression (Fig 1A). We stratified pancreatic cancer samples with differential UNR expression from negative to highly positive (Fig 1B–1E). All samples that stained positive exhibited a cytoplasmic expression pattern and some diffuse membrane localisation (Fig 1C–1E).

Subsequently, the association between UNR expression and outcome was assessed. Interestingly, it was observed that patients with negative/low or medium expression had similar behaviour according to progression-free survival, while patients with high expression clearly presented a better outcome ($P = 0.028$; Fig 2A). Therefore, high expression was established as cut-off point yielding two groups, with high- and low-risk according to low or high UNR expression, respectively.

Survival analysis performed with low or high expression of UNR showed shorter progression-free survival in the arm with low UNR expression ($P = 0.010$) (Fig 2B). Mean progression-free

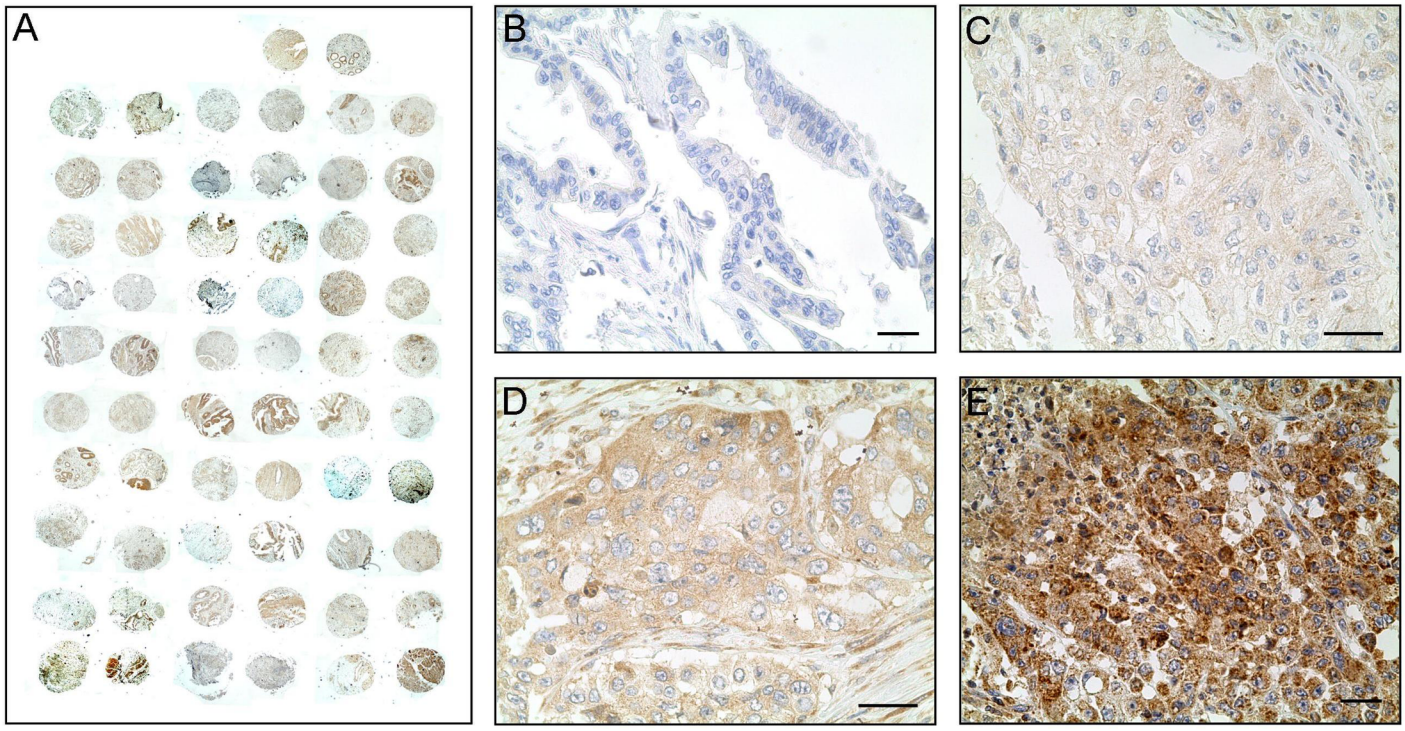

Fig 1. UNR immunostaining. A) The TMA slide contained 62 tumor tissue cores (2 cores per patient) and was immunostained with the anti-CSDE1 antibody. Representative images of tumor samples exhibiting negative UNR expression (B), low (C), medium (D) and high UNR expression (E). Scale bar: 10 μm.

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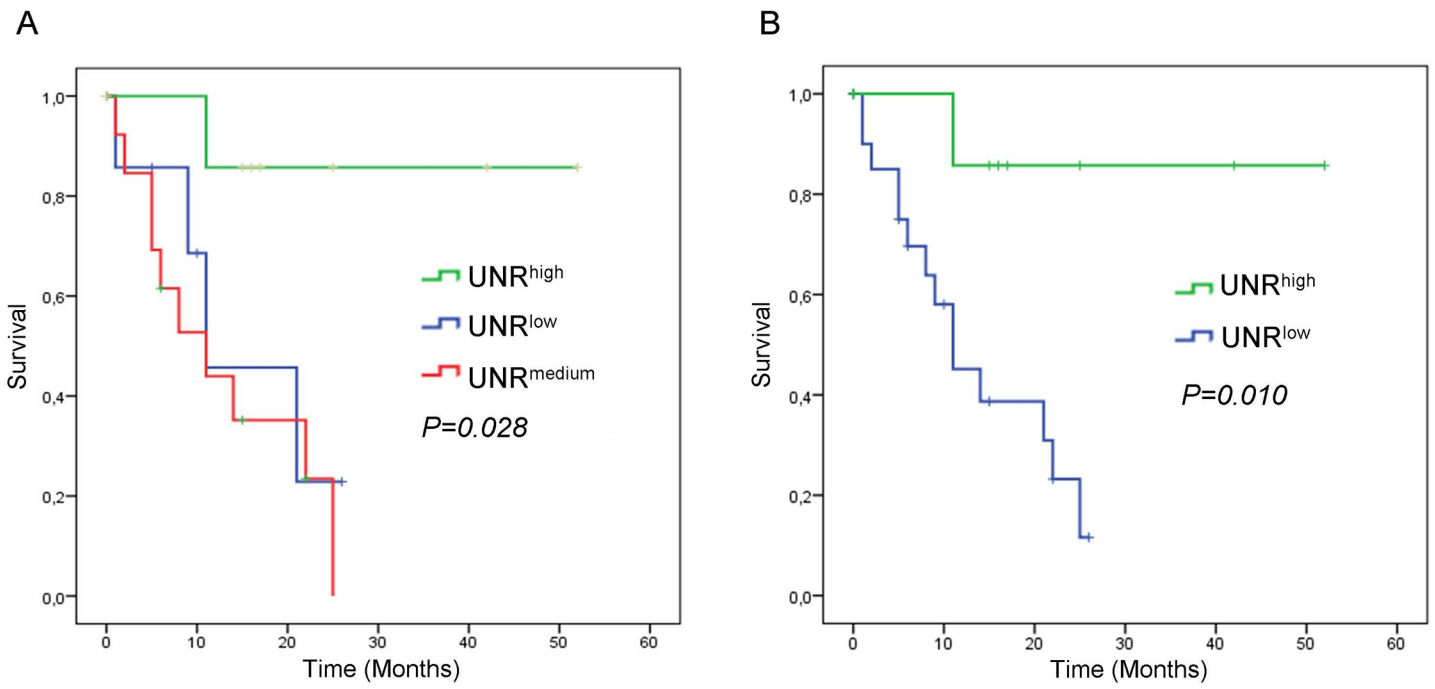

Fig 2. Kaplan-Meier analysis for progression-free survival after surgery based on UNR expression levels in low-grade resectable PDAC patients. A) Survival curves according to UNR expression stratified in tertiles. B) Survival curves of PDAC patients according to low or high UNR expression.

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survival for patients expressing low levels of UNR was 13 months (range 9–17 months), while mean survival for those expressing high levels of UNR was 46 months (35–56 months) (Table 2). Median revealed that patients with low levels of UNR took 11 months to experience disease recurrence (range 5–17 months), while the median was not reached in the case of patients with high UNR levels (Table 2).

In order to compare the potential prognosis value of UNR expression with the other clinical variables we performed a Cox proportional hazards model. The univariate analysis for progression-free survival confirmed that patients with low expression of UNR showed higher risk of recurrence after surgery compared to those with high expression of UNR (HR = 8.914; P = 0.036) (Table 3). Moreover, UNR expression remained the only significant variable in this analysis.

Overall survival was analysed as a secondary endpoint. However, we did not find any statistically significant difference between arms with high or low UNR expression levels (P = 0.429; data not shown).

Table 2. Progression-free survival (months) according to UNR expression.

		Mean			Median		
		95% CI			95% CI		
UNR	**Months**	**Lower**	**Upper**	**Months**	**Lower**	**Upper**	***P-value***
Low	13.576	9.453	17.700	11.000	4.925	17.075	0.010
High	46.143	35.514	56.771	-	-	-	

CI: confidence interval

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Table 3. The effect of the molecular and clinical variables on progression-free survival in resectable low-grade pancreatic cancer patients.

		Univariate		
		95% CI		
	HR	Lower	Upper	*P-value*
Age				0.588
> 65 years *vs* < 65 years	1.313	0.490	3.518	
Sex				0.540
Male *vs* Female	1.336	0.528	3.381	
Adjuvant treatment				0.329
No *vs* Yes	1.718	0.579	5.093	
Tumor size				0.926
>2 cm *vs* <2cm	1.050	0.373	2.959	
Stage				0.173
II *vs* I	2.540	0.571	11.306	
pT				0.341
T3 *vs* T1-T2	1.854	0.521	6.601	
pN				0.565
N1 *vs* N0	1.385	0.461	4.159	
Tumor location				0.263
Pancreas *vs* Others	1.924	0.611	6.053	
Vascular Invasion				0.728
Yes *vs* No	1.220	0.399	3.731	
Neural Invasion				0.728
Yes *vs* No	1.220	0.399	3.731	
Lymph nodes affected				0.312
Yes *vs* No	1.719	0.602	4.911	
UNR				0.036
Low *vs* High	8.914	1.159	68.584	

HR: hazard ratio; CI: confidence interval; *vs*: versus

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To verify if expression of UNR/*CDSE1* could be related to any clinico-pathological variable a crosstab was performed thereafter (Table 4). Here, there were no statistically significant associations between UNR expression and all variables of the study. This analysis included gender ($P = 0.704$), age ($P = 1.000$), stage ($P = 0.150$), pT ($P = 0.185$), pN ($P = 0.418$), tumor size ($P = 1.000$), lymph-node involvement ($P = 0.418$), neural invasion ($P = 0.185$) and positive margins of resection ($P = 1.000$). Interestingly, low UNR expression showed a high trend towards significance with vascular invasion ($P = 0.077$) (Table 4).

Since the *CDSE1* locus is only 150 nucleotides upstream of the *NRAS* gene and its regulation has been previously correlated with UNR expression [30], NRAS protein was also quantified by immunohistochemistry and a link between UNR/*CDSE1* and *NRAS* expression was evaluated. Nevertheless, no correlation was found between the expression levels of both proteins ($P = 0.903$). Additionally, a survival analysis performed with Kaplan-Meier plots confirmed the lack of association; instead, a high trend towards significance was found between *NRAS* expression and both progression-free survival ($P = 0.054$) and overall survival ($P = 0.092$) in this set of patients (data not shown).

Table 4. Association between UNR expression and clinico-pathological parameters.

	UNR^{low}	UNR^{high}	
Parameters	**N**	**N**	***P-value***
Gender			0.704
Female	12	4	
Male	10	5	
Age			1.000
< 65 years	9	3	
> 65 years	13	6	
Stage			0.150
I	3	4	
II	19	5	
pT			0.185
T1-T2	4	4	
T3	18	5	
pN			0.418
N0	7	5	
N1	14	4	
Size			1.000
< 2 cm	13	6	
> 2 cm	9	3	
Lymph nodes involved			0.418
No	7	5	
Yes	14	4	
Vascular Invasion			0.077
No	4	5	
Yes	18	4	
Neural Invasion			0.185
No	4	4	
Yes	18	5	
Positive margins			1.000
No	20	8	
Yes	2	1	

N: number of patients

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Survival analysis according to UNR/*CSDE1* expression in PDAC validation cohorts

We next analysed survival according *CDSE1 mRNA* expression on two independent datasets of pancreatic cancer patients used as validation sets. One cohort was taken from The Cancer Genome Atlas (TCGA) using the cBioPortal Interface [34, 35], and the other was taken from Gene Expression Omnibus (GEO) database.

Patients from TGCA that presented non-cancer related death, incomplete resections (R1), neuroendocrine origin, high-grade of differentiation, stage III/IV, *CDSE1* mutations, treated with neoadjuvant chemotherapy, or missed *CDSE1* expression data or clinical/pathological information where excluded from the study (S1A Fig). Progression-free survival analysis of 47 eligible patients showed that patients with high *CDSE1* expression presented better survival compared to low *CDSE1* expression cases ($P = 0.009$; median survival of 28 months vs. 14

months, respectively) (S1B Fig). Overall survival analysis with 60 patients did not achieve statistical significance; however, a high trend toward significance was found between patients with high and low *CDSE1* expression ($P = 0.056$). Here, patients with high *CDSE1* expression presented longer overall survival (median survival of 30 months, compared to 20 months for patients with low CSDE1 expression) (S1C Fig).

All patients from GEO database were included in the study except for those with no survival information (n = 3). As this dataset lacks information on pathology, we included all patients with no inclusion/exclusion criteria. Perhaps not surprisingly, given that patients were analysed independently of grade of differentiation, stage, treatment or positive resection margins, overall survival analysis revealed no statistical significance between high or low *CDSE1* expression ($P = 0.129$). However, patients with high *CDSE1* expression showed longer median overall survival than patients with low *CDSE1* expression (median overall survival 21 months vs. 13 months, respectively) (S2 Fig). Altogether, the results from both validation sets support the observation that high UNR/*CDSE1* expression correlates with better outcome in resectable PDAC patients.

The expression of *CDSE1* is associated to the immunogenic molecular subtype of pancreatic cancer

The mRNA expression profile of 186 pancreatic cancer patients from the TGCA dataset was correlated with the expression of *CSDE1* using Spearman and Pearson tests. Here, the expression of *CDSE1* and *NRAS* transcripts correlated (Spearman = 0.63; Pearson = 0.66) (Fig 3). Interestingly, we found a moderate correlation between *CDSE1* and *TLR4* (Spearman = 0.49; Pearson = 0.44), *TLR7* (Spearman = 0.41; Pearson = 0.37), and *TLR8* expression (Spearman = 0.41; Pearson = 0.33) (Fig 3). The expression of these Toll-like receptor genes has been associated with the pancreatic cancer immunogenic subtype defined by Bailey *et al.* [37]. It was reported that patients classified under the immunogenic subtype present a better prognosis compared to the other subtypes: ADEX (abnormally differentiated endocrine exocrine), progenitor and squamous subtype (median survival of 30.0, 23.7, 25.6 and 13.3 months, respectively) [37]. On the other hand, *CDSE1* expression showed negative correlation with progenitor subtype genes such as *PDX1* (Spearman = -0.20; Pearson = -0.14), *FOXA3* (Spearman = -0.28; Pearson = -0.19), *MNX1* (Spearman = -0.34; Pearson = -0.17) and *FOXA2* (Spearman = -0.40; Pearson = -0.18) (Fig 3).

Overall, consistent with our immunohistochemistry data, these *in silico* analyses support the notion that UNR/*CDSE1* expression predicts better outcome in resectable PDAC patients. Further analyses using larger patient cohorts should be performed to confirm these promising pilot results.

Discussion

PDAC is rare, although due to its poor clinical outcome it is the fourth leading cause of cancer death. A demographic report showed that the incidence of this cancer is rising worldwide [2], possibly associated with an increase in consumption of sugar, high-carbohydrate-content foods, red and processed meat or obesity [38–40]. The most effective standard treatment consists of pancreatectomy performed by Whipple procedure [41]. Oncology guidelines are useful to manage this kind of patients [42, 43]. Although treatment options for this cancer are increasing [44–46], mortality continues around 74% within the first year of diagnosis. It is therefore imperative to find new treatments, predictive tools and translational prognostic biomarkers to personalise the therapy and improve survival [47].

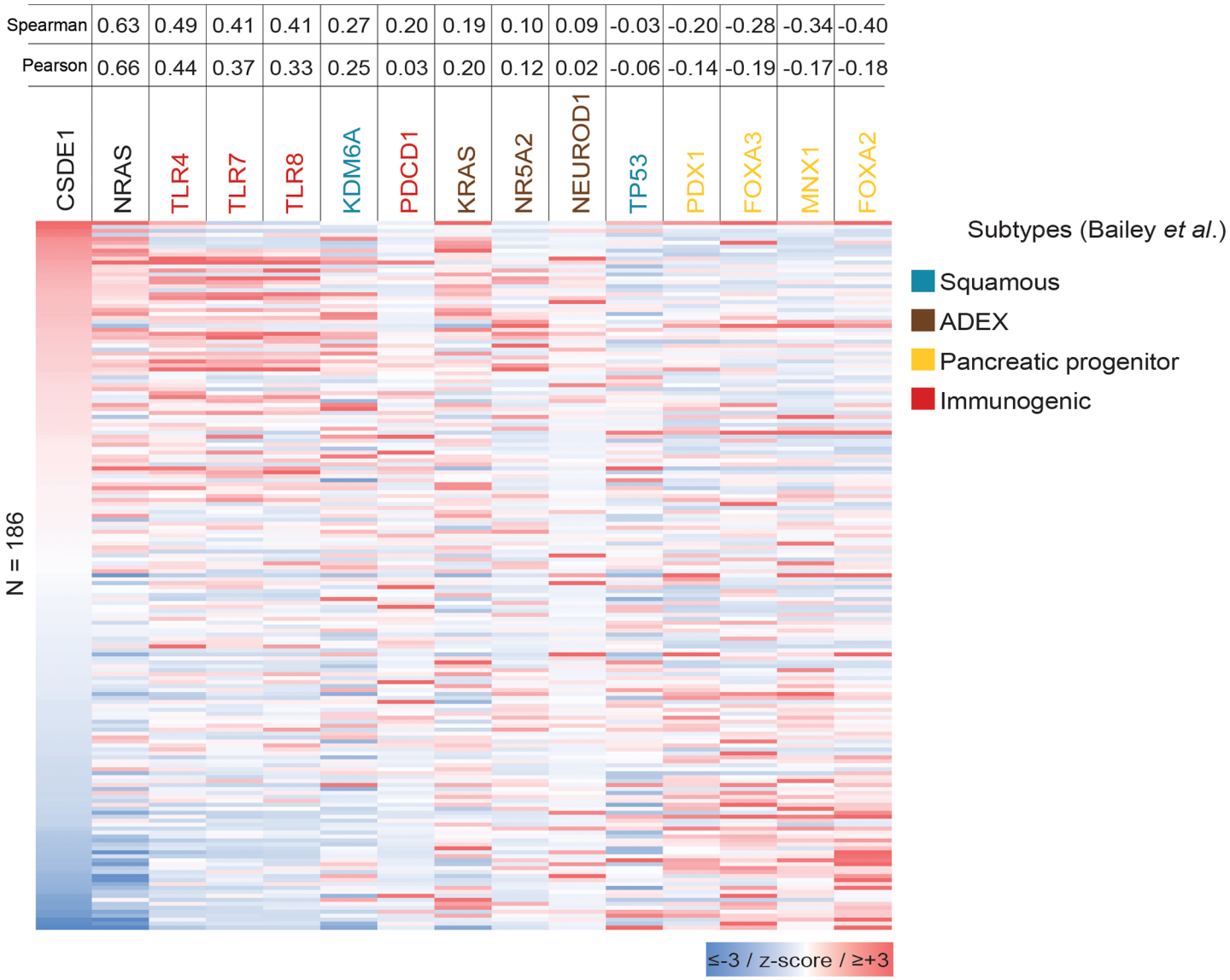

Fig 3. Heatmap comparison of Z-scores that correlated with *CSDE1* expression. Spearman and Pearson analyses show correlation between *CSDE1* expression and the main genes of Bailey´s molecular subtypes of pancreatic cancer.

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Post-transcriptional gene regulation is a rapid and efficient way to adjust the proteome of a cell to environments in constant variation. RBPs regulate post-transcriptional gene expression during biological processes such as cell proliferation, differentiation, invasion, metastasis, and apoptosis [20]. In addition, RBPs bind hundreds of mRNAs to form complex networks that are crucial for tumor development. UNR is an RBP related with multiple processes, such as apoptosis [48], stem-cell differentiation [24] and the migration of pre-cerebellar neurons [49]. Regarding cancer, UNR has been considered a pro-oncogenic factor for its role in stabilising c-fos mRNA and simulating the translation of c-myc mRNA [25, 26], and promoting melanoma metastasis [27]. However, upregulation of UNR is not always associated to tumor progression, indicating that the precise role of UNR in cancer depends on context. For example, overexpression of the *HEPSIN* oncogene in prostate cancer [28] downregulates the expression and IRES activity of UNR [29]. Consistent with a protective effect of UNR, we describe here an

association between low levels of UNR and poor clinical outcome of PDAC patients. It has been described an association between *CSDE1* mRNA and protein expression along cell cycle [50, 51]. Thus, we analysed two independent datasets based on mRNA expression profile, and *CSDE1* expression results were in agreement with our previous findings. These results are in line with those of Cornelis *et al*. reporting that a constitutive high expression of UNR becomes cytotoxic and leads to cell death [52]. In the same vein, UNR-deficient murine embryonic stem cells display resistance to apoptosis after irradiation [48]. Thus, in certain cancer types UNR may act to suppress tumor formation.

The available expression profile of 186 pancreatic cancer patients from TGCA database allowed us to correlate *CDSE1* expression to genes associated with specific molecular subtypes of pancreatic cancer. In this analysis, *CDSE1* presented a moderate correlation with genes involved in Toll-like receptor signalling pathway. This pathway mediates innate immunity and triggers pro-inflammatory signalling cascades [53]. The correlation between *CDSE1* and *TLR4*, *TLR7* or *TLR8* expression suggests that PDAC patients with high UNR/*CDSE1* expression may present a less aggressive tumor phenotype, more susceptible to be cleared by the immune response [54, 55].

The *CDSE1* and *NRAS* loci are located close together in the genome, with an intergenic distance of only 150 nucleotides. This special location raised the possibility of transcriptional interference between both genes. Indeed, such interference was found in mouse tissues, where deletion of the *CSDE1* promoter led to an increase in *NRAS* mRNA accumulation [29]. Contrary to results in the mouse, however, we find no evidence for an anti-correlation in human tumor samples. Rather, we find a direct correlation between *CSDE1* and *NRAS* mRNA levels in PDAC samples from the TGCA database. Furthermore, this correlation is not maintained at the protein level, as we found no relationship between CSDE1 and NRAS protein levels by immunohistochemistry. Therefore, the protective role of CSDE1 is not explained by simple down-regulation of NRAS, and must rely on other targets.

Future experiments should be directed towards the identification of these targets. In the meantime, our results provide a proof-of-concept study supporting UNR/*CDSE1* expression as a potential biomarker for PDAC prognosis.

Conclusions

Here, we describe the association between low UNR expression and poor outcome of low-grade resectable PDAC patients. Low expression of UNR showed a statistical trend when it was associated with vascular invasion and other clinico-pathological characteristics like neural invasion, pT and stage, indicating UNR loss as a feasible factor to induce malignant phenotype, and therefore, a poor outcome event in PDAC development. Furthermore, UNR expression was associated with immunogenic phenotype of pancreatic cancer. Based on these findings, we propose UNR/CSDE1 as an independent prognostic biomarker for resectable pancreatic cancer.

Supporting information

S1 Fig. Survival analysis of TGCA validation set according *CSDE1* expression. A) Flow chart of the selected population and exclusion criteria. B) Kaplan–Meier analysis for progression-free survival and overall survival (C) based on *CSDE1* mRNA expression level.
(TIF)

S2 Fig. Kaplan-Meier analysis of overall survival of GEO validation set (GSE28735) according *CSDE1* expression.
(TIF)

S1 Table. Clinical and pathological information of patients recruited in the study.
(DOC)

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SUPPLEMENTARY INFORMATION

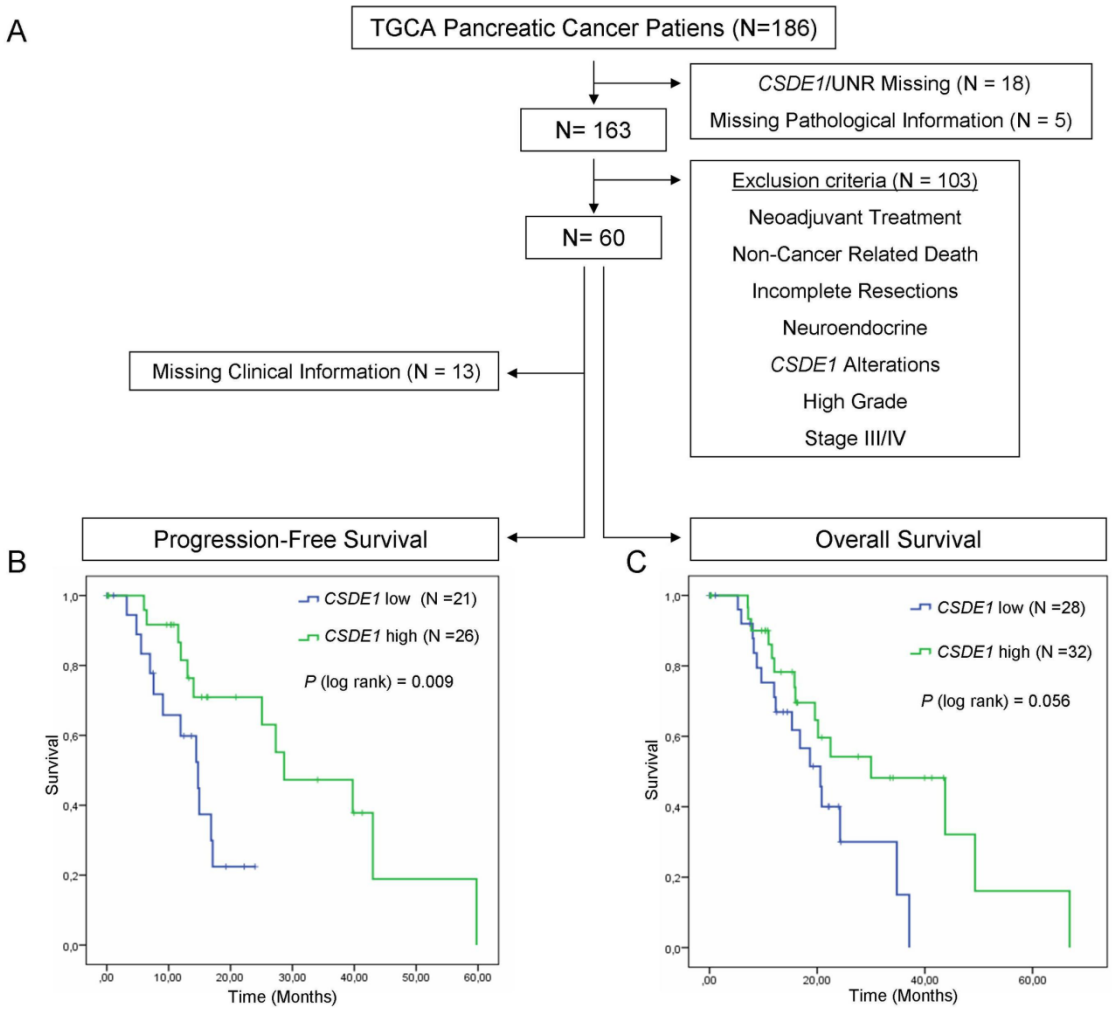

Figure S1: Survival analysis of TGCA validation set according *CSDE1* expression. A) Flow chart of the selected population and exclusion criteria. B) Kaplan–Meier analysis for progression-free survival and overall survival (C) based on *CSDE1* mRNA expression level.

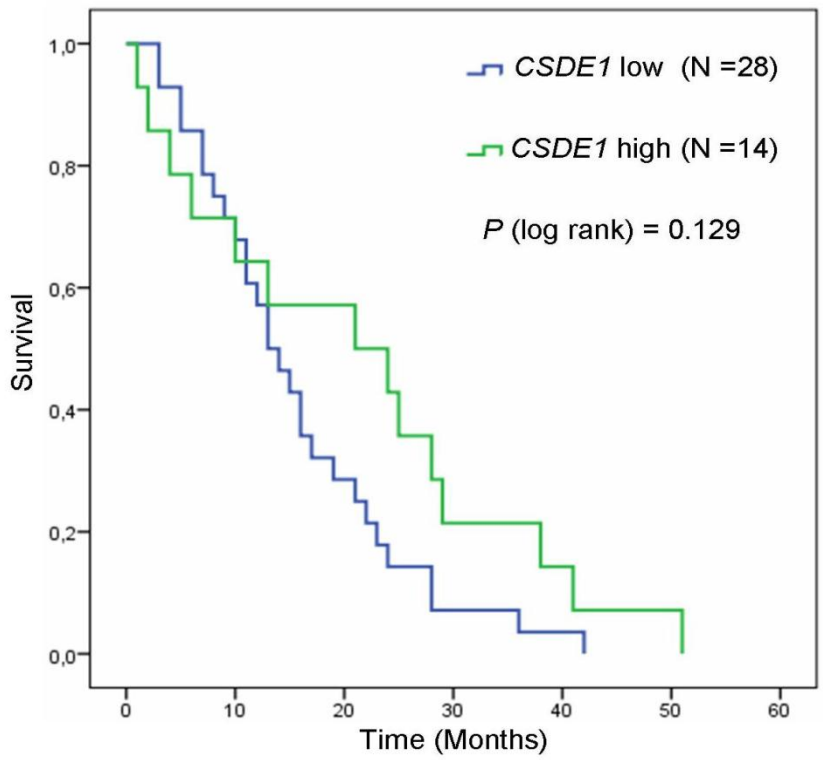

Figure S2: Kaplan-Meier analysis of overall survival of GEO validation set (GSE28735) according CSDE1 expression.

Table S1. Clinical and pathological information of patients recruited in the study.

Sample	Age	Gender	Tumor size	Stage	pTN	Origin	Adjuvant treatment	R	Vascular invasion	Neural invasion	UNR expression	PFS (months)	Progression Event	OS (months)	Survival event
1	64	Male	< 2 cm	1A	T1N0	Ampulla	None	R0	No	No	High	0	No	0	Dead
2	70	Female	< 2 cm	1A	T1N0	Ampulla	None	R0	No	No	High	11	Yes	36	Alive
3	73	Male	> 2 cm	2B	T3N1	Biliar duct	Gemcitabine	R0	Yes	Yes	High	15	No	20	Alive
4	77	Male	< 2 cm	2A	T3N0	Biliar duct	None	R1	No	Yes	High	0	No	0	Alive
5	79	Male	< 2 cm	1A	T1N0	Pancreas	None	R0	Yes	Yes	High	52	No	52	Alive
6	60	Female	> 2 cm	2B	T3N1	Ampulla	Gem + RT	R0	Yes	Yes	High	42	No	42	Alive
7	54	Female	< 2 cm	2B	T3N1	Biliar duct	Gemcitabine	R0	No	No	High	25	No	25	Alive
8	69	Male	> 2 cm	2B	T3N1	Biliar duct	None	R0	Yes	Yes	High	17	No	17	Alive
9	77	Female	< 2 cm	1A	T1N0	Pancreas	None	R0	No	No	High	16	No	16	Alive
10	65	Male	< 2 cm	1A	T1N0	Pancreas	None	R0	No	No	Medium	6	No	6	Alive
11	37	Male	> 2 cm	2B	T3N1	Ampulla	Gem + RT	R0	Yes	Yes	Medium	5	Yes	5	Alive
12	75	Male	< 2 cm	2B	T3N1	Ampulla	None	R0	No	No	Medium	5	Yes	7	Dead
13	66	Female	< 2 cm	2B	T2N1	Biliar duct	Gem + RT	R0	Yes	Yes	Medium	11	Yes	37	Alive
14	51	Female	> 2 cm	2B	T3N1	Pancreas	Gem + RT	R1	Yes	Yes	Medium	22	No	22	Alive
15	80	Female	< 2 cm	2B	T3N1	Biliar duct	None	R0	Yes	Yes	Medium	0	No	0	Dead
16	51	Female	< 2 cm	2A	T3N0	Pancreas	Gemcitabine	R0	Yes	Yes	Medium	8	Yes	11	Alive
17	62	Female	< 2 cm	1B	T2N0	Ampulla	None	R0	No	No	Medium	15	No	15	Alive
18	76	Female	< 2 cm	2B	T3N1	Pancreas	None	R0	Yes	Yes	Medium	2	Yes	3	Dead
19	59	Male	< 2 cm	2B	T3N1	Biliar duct	None	R0	Yes	Yes	Medium	22	Yes	54	Alive
20	74	Female	< 2 cm	2B	T3N1	Ampulla	None	R0	No	No	Medium	14	Yes	51	Alive
21	76	Male	< 2 cm	1B	T2N0	Ampulla	None	R0	Yes	Yes	Medium	25	Yes	32	Alive
22	82	Male	> 2 cm	2B	T3N1	Biliar duct	None	R0	Yes	Yes	Medium	1	Yes	13	Dead
23	74	Male	> 2 cm	2A	T3Nx	Biliar duct	None	R0	Yes	Yes	Medium	6	Yes	8	Alive
24	56	Male	< 2 cm	2A	T3N0	Pancreas	None	R0	Yes	Yes	Low	11	Yes	11	Alive
25	68	Male	> 2 cm	2B	T3N1	Pancreas	None	R0	Yes	Yes	Low	5	No	5	Alive
26	70	Female	> 2 cm	2A	T3N0	Pancreas	Gemcitabine	R0	Yes	Yes	Low	26	No	26	Alive
27	79	Female	< 2 cm	2B	T3N1	Pancreas	None	R1	Yes	Yes	Low	10	No	10	Alive
28	60	Female	< 2 cm	2B	T3N1	Pancreas	None	R0	Yes	Yes	Low	0	No	0	Alive

29	66	Male	> 2 cm	2B	T3N1	Pancreas	Gemcitabine	R0	Yes	Yes	Low	21	Yes	35	Alive
30	57	Female	> 2 cm	2B	T3N1	Biliar duct	Gemcitabine	R0	Yes	Yes	Low	1	Yes	6	Dead
31	78	Female	> 2 cm	2A	T3N0	Ampulla	None	R0	Yes	Yes	Negative	9	Yes	11	Alive

cm: centimetres; Gem: Gemcitabine; RT: radiotherapy; R: resection margins; PFS: progression-free survival; OS: overall survival.

Discusión General

El CaPa es una neoplasia maligna extremadamente letal que actualmente presenta un resultado clínico muy pobre debido a su diagnóstico tardío con enfermedad metastásica. Aquí, el diagnóstico temprano es crucial para aumentar la supervivencia de los pacientes. Por lo tanto, los biomarcadores moleculares desempeñarán un papel importante en el manejo futuro de esta neoplasia. Hasta el día de hoy, los únicos biomarcadores aprobados por la Administración de Alimentos y Medicamentos (FDA) para CaPa son los niveles preoperatorios de CA19-9; sin embargo, la aplicabilidad de este biomarcador ha sido cuestionada debido al hecho de que la obstrucción biliar también puede aumentar los niveles de CA19-9, sin mencionar que hasta el 10% de la población no puede sintetizar este antígeno(14). Por lo tanto, se necesitan nuevos biomarcadores que combinen alta sensibilidad y especificidad en el manejo clínico del CaPa.

Los cambios pleiotrópicos en el transcriptoma son una característica clave de las células cancerosas(93). Las proteínas de unión a ARN (RBPs) son relevantes porque forman parte de los mecanismos reguladores postranscripcionales. Estas proteínas reguladoras de ARN están formadas por ribonucleoproteínas (RNP) que interactúan con otros elementos, ARN no codificantes, metabolitos y elementos de secuencia no traducidos que se encuentran dentro de los ARNm. Estos complejos RNP controlan la expresión de múltiples ARNm que pasan a proteínas relacionadas funcionalmente desde el proceso de transcripción a traducción, lo que permite que la célula responda a varios estímulos con una agilidad tan grande asegurando la homeostasis celular(29). En este sentido, las RBPs están emergiendo como moduladores críticos de cada sello distintivo del cáncer, y aún se sabe muy poco sobre sus funciones y objetivos moleculares relacionados con el cáncer(93).

Se han descubierto dos nuevas proteínas de unión a ARN llamadas PIWI y UNR, sus expresiones se encontraron en varios tipos de tumores. Las proteínas PIWI se unen y regulan pequeños ARN no codificantes para inhibir la expresión génica, mientras que UNR es una proteína que regula los ARNm y a otras proteínas controlando la traducción. Debido a las multiples funciones que tienen estas proteínas, estos factores pueden proporcionar nuevas perspectivas en la práctica clínica del cáncer en general y del CaPa en particular.

1. **PIWIL1 and PIWIL2 presentan niveles de expresión casi indetectables tanto en líneas celulares tumorales derivadas de cáncer de páncreas como en línea celular pancreática normal.**

Las proteínas PIWI están involucradas en la división de células madre, gametogénesis, especificación de línea germinal y silenciamiento de ADN(91,94). Para estudiar mejor su función en CaPa, en primer lugar hemos evaluado la expresión de los cuatro miembros de la familia PIWI en líneas celulares derivadas del CaPa y una línea celular pancreática normal utilizada como control. Curiosamente, tanto PIWIL1 como PIWIL2 presentaron niveles de expresión casi indetectables en todas las líneas celulares. Este hecho podría explicarse por la presencia de islas CpG en la región promotora de PIWIL1(95) y PIWIL2(96). Se ha descrito que la desregulación de PIWIL1 y PIWIL2 por la hipermetilación de la isla CpG del promotor se ha observado en otros tipos de tumores como el cáncer de pulmón de células no pequeñas y testiculares(74). Dado que también encontramos niveles bajos de PIWIL1 y PIWIL2 en la línea celular normal pancreática, este evento parece no ser exclusivo de las células tumorales. De hecho, estos genes juegan papeles cruciales en la espermatogénesis, y su regulación negativa perjudica el desarrollo de células germinales que podrían asociarse con la infertilidad masculina (97).

2. **La expresión de PIWIL1 no tuvo impacto en la supervivencia del cáncer de páncreas tanto en los niveles de proteína como ARNm.**

Debido a que la expresión de PIWIL1 y PIWIL2 fué casi indetectables en líneas celulares, nos preguntamos si dicha expresión también estaría disminuida en muestras de pacientes. Por ello nos centramos en el estudio de la expresión de las proteínas PIWIL1 y PIWIL2 para estudiar su valor pronóstico en CaPa y ayudar a los médicos en el manejo del paciente.

Se ha descrito que PIWIL1 exhibe un valor pronóstico pobre en el sarcoma de tejidos blandos(73), carcinoma de células escamosas esofágicas(98), cáncer colorrectal(67,99), gliomas(100), carcinoma hepatocelular humano(101,102), cáncer gástrico(69,103), cáncer de pulmón(54), cánceres ginecológicos(59) o carcinoma de células renales(57,104). La alta expresión de PIWIL1 también indica un mal pronóstico

en cáncer colorrectal, lo que sugiere que PIWIL1 es un marcador molecular importante para predecir mal pronóstico en esta enfermedad(99). PIWIL1 junto con piR-823 juegan un papel importante en la patogénesis de las células cancerosas renales. Sin embargo, es la baja expresión de PIWIL1 la que se asocia con un fenotipo tumoral más agresivo y con una peor supervivencia en pacientes con cáncer de células renales, lo que indica que PIWIL1 puede servir como factor protector(57).

Sólo un estudio informó de la importancia pronóstica de PIWIL1 en CaPa determinada por los niveles de ARNm y proteína(105). En este estudio así como en nuestros resultados, la expresión de PIWIL1 tanto en los niveles de proteína como ARNm no tuvieron un impacto en la supervivencia. Sin embargo, en ese estudio, la expresión de ARNm alterada (tanto alta como baja) presentó una supervivencia más corta que aquellos pacientes con niveles intermedios de ARNm de PIWIL1 (P=0,034)(105). Desde nuestro punto de vista, el uso de la expresión intermedia de ARNm sería bastante limitado en la práctica clínica. Por esta razón, centramos nuestro estudio en los niveles de expresión de proteínas evaluados por tinción inmunohistoquímica. Sin embargo, la expresión de la proteína PIWIL1 no tuvo suficiente poder estadístico para considerarse un biomarcador pronóstico, aunque mostró una asociación con pacientes masculinos y una tendencia con el origen del tumor pancreático.

3. La proteína PIWIL2 exhibió un mayor potencial de pronóstico para predecir una supervivencia tanto libre de progresión como global más largas.

La expresión de PIWIL2 también se ha asociado con el desarrollo tumoral de cáncer de ovario(59), carcinoma de células renales(57), cáncer de mama(60), cáncer gástrico(69) y cáncer colorrectal(106). En glioma, la alta expresión de PIWIL2 correlaciona con mal pronóstico(58). Sin embargo, la baja expresión de PIWIL2 se asocia con peor superviviencia en carcinoma de células renales(57). El efecto tumorigénico de la expresión de PIWIL2 parece ser bastante controvertido y sigue sin estar claro. En este sentido, la modulación epigenética de la expresión de las proteínas PIWI podría jugar un papel crucial y justificar su papel ambivalente en la tumorigénesis a través de la regulación positiva de las metiltransferasas de ADN(107). Dado su papel

en tumorigénesis, también evalué el potencial valor pronóstico de PIWIL2 en nuestra cohorte de pacientes.

Dado que *The Human Protein Atlas Project* sólo proporciona la expresión de ARNm de PIWIL2, nosotros describimos por primera vez el perfil de expresión de PIWIL2 a nivel de proteína. Para ello, usamos tejidos de testículos humanos para determinar la mejor concentración de anticuerpos y una vez puesta a punto, evaluamos PIWIL2 en nuestra serie de muestras. Los análisis de supervivencia en nuestra cohorte de pacientes según la expresión de la proteína PIWIL2 revelaron que la falta de PIWIL2 es un evento negativo en el pronóstico y reduce tanto la supervivencia libre de progresión como la supervivencia global. Este efecto también fue respaldado por el análisis multivariado de Cox para la supervivencia global, donde la expresión de PIWIL2 siguió siendo el único factor molecular significativo. Además, se observó una asociación estadística entre los bajos niveles de expresión de PIWIL2 y el estadío T más alto, y una alta tendencia hacia la significancia con la invasión vascular, invasión neural y estadios más avanzados de la enfermedad, lo que apoya el papel de que los bajos niveles de expresión de PIWIL2 tienen un efecto perjudicial en la progresión y el desarrollo de CaPa.

Varios estudios apoyan nuestro resultado. En cáncer colorrectal se observó una disminución de la expresión de ARNm de PIWIL2 en comparación con tejidos no transformados(108). También se ha encontrado que la expresión de la proteína PIWIL2 está regulada negativamente en muestras de cáncer de pulmón de células no pequeñas en comparación con el tejido normal ($P<0,001$)(74). Además, los bajos niveles de ARNm de PIWIL2 han sido estadísticamente significativos en las muestras de carcinoma de mama en comparación con los tejidos de mama normales ($P<0.001$)(109).

4. PIWIL1 y PIWIL2 se asociaron al subtipo molecular progenitor de cáncer pancreático tanto en los niveles de expresión de ARNm como de proteína.

Varios artículos demostraron que las proteínas PIWI están asociadas a varios genes implicados en la regulación del ciclo celular, la apoptosis, la proliferación y la migración de las células tumorales. Las proteínas PIWI regulan varias vías moleculares

a través de mediadores clave en diferentes neoplasias. Por ejemplo, PIWIL1 regula la apoptosis y la progresión del ciclo celular a través de P21, Ciclina D1, BCL-2 y BAX, y la migración a través de la expresión de MMP-2 y MMP-9, en células de glioma, por lo que podría usarse como un marcador molecular para los gliomas malignos en el diagnóstico y la evaluación del pronóstico(100). Por el contrario, la expresión de PIWIL1 desregula MMP-2 y MMP-9 e inhibe la proliferación y la capacidad de migración de las células de leucemia mieloide crónica (110). En el cáncer gástrico, PIWIL1 se ha asociado con los genes *OCK2, ZNF503, PDE4D, ABL1, ABL2, LPAR1, SMAD2, WASF3 y DACH1*(103), y ha mostrado una actividad reguladora de la transición epitelio-mesenquimal en el cáncer de endometrio(103). PIWIL1 también es capaz de regular OCT4, que es un factor asociado al mal pronóstico y al estadío metastásico en el cáncer colorrectal(108).

PIWIL2 regula BCL-XL y STAT3, y su regulación negativa suprime la expresión de proteínas que desencadenan la cascada de apoptosis y aumenta la sensibilidad al cisplatino(58). En cáncer de pulmón de celulas no pequeñas, PIWIL2 aumenta la expresión de CDK2 y Cyclina A, además de aparecer como un potencial factor pronóstico y terapéutico(56). Se ha observado una correlación positiva entre PIWIL2 y el marcador celular indiferenciado SOX2 en tejidos de cáncer colorrectal, lo que indica que PIWIL2 juega un papel directo en la progresión del CRC(108). Curiosamente, Chen *et al.* sugirió que la expresión ectópica de PIWIL2 puede contribuir al desarrollo y la proliferación de células madre pre-cancerosas con potencial de diferenciación benigna y maligna(111).

En 2016, Bailey *et al.* describió 4 subtipos moleculares del CaPa: (1) escamoso; (2) progenitor pancreático; (3) inmunogénico; y (4) exocrino-endocrino aberrantemente diferenciado (ADEX); además identificó sus distintos pronósticos y oportunidades para el desarrollo terapéutico(112). Para entender mejor las vías moleculares de las proteínas PIWI, hicimos una correlación con los genes más significativos de cada subtipo molecular. Aquí describimos que la mayoría de los factores significativos asociados con el subtipo molecular progenitor tienen una correlación positiva con PIWIL1 y PIWIL2 a nivel de ARNm. Además, procedimos a validar estos resultados de ARNm a nivel de proteína y observamos como la expresión

de proteínas de PIWIL2 se correlacionó positivamente con MUC17 y se asoció significativamente con HNF4A, mientras que la expresión de PIWIL1 se asoció significativamente con la expresión de la proteína PDX1. Este hecho apoya el vínculo entre estas dos proteínas PIWI y el subtipo molecular progenitor pancreático, que está involucrado en el desarrollo temprano del endodermo pancreático y está relacionado con la diabetes de inicio en la madurez(112).

5. PIWIL3 y PIWIL4 son factores cruciales en la regulación de la motilidad celular, el mantenimiento de las células madre y la resistencia a la quimioterapia tanto en las células tumorales como en las pancreáticas sanas.

Comparando con PIWIL1 y PIWIL2, hay pocos estudios centrados en PIWIL3 y PIWIL4 en relación con el cáncer. El papel de PIWIL3 y PIWIL4 en la tumorigénesis es bastante controvertido. Algunos estudios han demostrado la expresión de estas proteínas con características oncogénicas. Un artículo destacó que PIWIL3 y PIWIL4 presentaban potencial oncogénico en varios tipos de cánceres(41). Además, PIWIL3 participa en la progresión y metastásis de mieloma múltiple(61). Por el contrario, PIWIL3 exhibió un efecto protector en las células de glioma(63), y también, se ha encontrado una baja expresión de PIWIL4 en células tumorales de carcinoma hepatocelular(72), cáncer de mama(70) y cáncer de pulmón de células no pequeñas(74). Por lo tanto, el papel de PIWIL3 y PIWIL4 en el inicio y desarrollo del tumor aún no está claro. Dado que la expresión de PIWIL3 y PIWIL4 no se ha estudiado en CaPa, nos hemos centrado en evaluar el papel de la expresión de PIWIL3 y PIWIL4 en las células pancreáticas y en estudiar su potencial pronóstico.

En primer lugar, evaluamos la expresión proteica de las proteínas PIWI en las líneas celulares derivadas de CaPa y en una línea celular pancreática normal. El resultado mostró niveles de proteína más altos de PIWIL3 y PIWIL4 y un patrón de expresión diferencial en todas las líneas celulares, que incluyó la línea celular no tumoral. Este primer intento implicaba que PIWIL3 y PIWIL4 podrían no actuar como un oncogén en CaPa. Para esto, decidimos evaluar su papel con experimentos funcionales en líneas celulares tumorales y en la línea celular no tumoral. En nuestros experimentos funcionales, diseñados para evaluar la motilidad celular, la

quimiorresistencia y el fenotipo indiferenciado, pudimos evaluar la respuesta celular a la desregulación de PIWIL3 y/o PIWIL4. Además, la inclusión de una línea celular no tumoral en estos experimentos nos llevó a discernir entre un verdadero papel oncogénico y una función celular normal.

Los resultados demostraron que PIWIL3 y PIWIL4 reducían la motilidad tanto de las células tumorales como de las normales a través de una reducción en el fenotipo mesenquimal a favor del fenotipo epitelial. Esta reducción de la motilidad celular por el silenciamiento de PIWIL4 se ha descrito previamente en células de cáncer de mama a través de un deterioro de Vimentina y N-Cadherina(70). Sin embargo, este estudio sólo proporcionó evidencia de retraso de la migración en la línea celular tumoral MCF7, pero no en una línea celular no tumoral. Entonces, aún se desconoce si la regulación por disminución de PIWIL4 afecta exclusivamente la motilidad celular de las células de cáncer de mama o si también afecta la motilidad de las células normales. En relación con la motilidad, otro estudio demostró que PIWIL2 regula las capacidades de invasión de las células de cáncer de próstata a través de la modulación de la expresión de la proteína TEM(113).

Desde un punto de vista clínico, esta conexión entre PIWIL3/PIWIL4 y la transición epitelio-mensénquima(TEM) debe manejarse con cuidado ya que la TEM es el mecanismo más crítico por el cual los tejidos adultos, incluidas las células β pancreáticas, se reparan después de lesiones inflamatorias, tóxicas o traumáticas (114–116).

Muchos trabajos han informado que las proteínas PIWI tienen la capacidad de regular los elementos transponibles para mantener la estabilidad genómica de las células madre(37). Este vínculo entre las proteínas PIWI y el fenotipo indiferenciado también se ha demostrado cuando la regulación negativa de las proteínas PIWI reduce la capacidad de regeneración de todo el cuerpo de ciertos organismos marinos(37). También, un estudio en cáncer de mama demostró que la desregulación de PIWIL2 era capaz de disminuir la proliferación y la supervivencia de las células madre del cáncer de mama a través de una disminución en los niveles de proteínas de STAT3, BCL-XL y Ciclina D1(109). En nuestros estudios funcionales, observamos una disminución del

fenotipo indiferenciado de las células pancreáticas, y encontramos un descenso en el número y tamaño de las esferas similares a las células madre pancreáticas después del silenciamiento de PIWIL3 y/o PIWIL4. Este resultado apoya el papel de PIWIL3/PIWIL4 tanto en la resistencia de las células a anoikis como en el mantenimiento del fenotipo indiferenciado a nivel tumoral y a nivel de las células normales que podrían formar parte del microambiente tumoral.

La expresión de las proteínas PIWI aumentó la resistencia a los medicamentos en el cáncer de pulmón de células no pequeñas(56) y en el cáncer de cuello uterino(68). Estos precedentes nos indujeron a pensar que quizás las proteínas PIWI pudieran estar implicadas en resitencia a fármacos en CaPa. Ello demostró que la inhibición de PIWIL3 y PIWIL4 aumenta el efecto de las quimioterapias más usadas en la práctica clínica (Gemcitabina sola o en combinación con Nab-Paclitaxel) contra CaPa. Sorprendentemente, la línea celular PL45 no mostró ningún efecto después de la inhibición de PIWIL3/PIWIL4 individual o combinada. Sin embargo, la falta de efecto en PL45 podría explicarse no sólo por sus mutaciones en *KRAS*, *TP53* o *DPC4*, que se encuentran comúnmente en CaPa, sino también por la mutación en el gen *BRCA2*, recientemente asociado a la quimiorresistencia en el CaPa(24).

Curiosamente, la línea celular no tumoral hTERT-HPNE mostró resistencia a la gemcitabina. Sin embargo, esta línea revirtió completamente su quimiorresistencia después del silenciamiento de PIWIL3 y/o PIWIL4 y aumentó significativamente el efecto de la Gemcitabina sola o en combinación con Nab-Paclitaxel. Sin embargo, esta respuesta farmacológica estadísticamente significativa no alcanzó en ningún momento ni un efecto aditivo ni sinérgico en comparación con el silenciamiento de proteínas individuales en presencia de un solo tratamiento o combinación. El hecho de que la desregulación de PIWIL3 y/o PIWIL4 incremente considerablemente la respuesta al fármaco en la línea celular normal hace que la modulación de PIWIL3 o PIWIL4 no sea adecuada para el potencial diseño de fármacos contra CaPa. Este efecto sobre las células normales podría implicar una mayor toxicidad y reacciones adversas, lo que podría comprometer la tolerabilidad y la seguridad de los pacientes.

Con el fin de explicar el vínculo entre estas dos proteínas PIWI y la quimiorresistencia, exploramos factores relacionados con la resistencia a la Gemcitabina o al Nab-Paclitaxel en CaPa.El factor alfa de hepatocitos (HNF4A) apareció rápidamente como un factor potencial que podría explicar este hallazgo. HNF4A es una proteína que se sobreexpresa en hepatocitos, enterocitos y células β pancreáticas. También asegura la expresión de genes intermedios necesarios para el metabolismo de la glucosa y los lípidos, y es necesario para la diferenciación celular(117). En CaPa, los altos niveles de expresión de HNF4A se han correlacionado con un mal pronóstico. Además, se ha descrito que HNF4A confiere quimiorresistencia en otros tipos de tumores como el cáncer de mama, donde ha sido el gen más sobrexpresado después de analizar las células que se han sometido a condiciones de hipoxia, lo que condujo a una mayor resistencia a la doxorrubicina(118). Además, el mecanismo de HNF4A para conferir quimiorresistencia a Gemcitabina es a través de su interacción directa de hENT1, que es el responsable de la absorción de Gemcitabina de las células tumorales(23). A primera vista, ni PIWIL3 ni PIWIL4 exhibieron una correlación con hENT1 a nivel de ARNm. Sin embargo, se encontró una alta tendencia entre PIWIL3 y HNF4A a nivel de proteína; pero lo que más nos llamó la atención es que se encontró una correlación estadísticamente significativa entre PIWIL4 y HNF4A tanto a nivel de ARNm como a nivel de proteína. Por lo tanto, estos resultados respaldan el papel de estas proteínas PIWI como factores cruciales para la regulación indirecta de la absorción la quimioterapia por parte de las células.

En resumen, en este artículo hemos descrito por primera vez la implicación de PIWIL3 y PIWIL4 en la motilidad celular a través de la modulación de la TEM de las células pancreáticas tumorales y no tumorales. Por otra parte, el papel de PIWIL3/PIWIL4 no parece ser exclusivo de la tumorigénesis y sugiere una función crucial en el mantenimiento de la homeostasis de los tejidos. Finalmente, la expresión de PIWIL4 parece estar relacionada con la quimiresistencia a través del factor HNF4A.

6. La baja expresión de PIWIL4 se asoció significativamente con una supervivencia libre de progresión y global más corta.

Según los resultados anteriores, PIWIL3 y PIWIL4 a pesar de estar implicados en la regulación de la motilidad celular, el mantenimiento de las células madre y la resistencia a los medicamentos, no presentan un papel oncogénico claro en CaPa. Sin embargo, es importante saber si las proteínas PIWIL3 y PIWIL4 se asocian con la superviviencia de los pacientes con CaPa. Por ello analizamos la supervivencia de los pacientes dependiendo de la expresión de PIWIL3 o PIWIL4 en las muestras por inmunohistoquímica. Los análisis de supervivencia revelaron que la baja expresión de PIWIL4 se asociaba significativamente con una supervivencia libre de progresión y global más corta. Por otra parte, la expresión de PIWIL3 no tuvo impacto en la supervivencia de los pacientes.

Estos resultados sugirieron un efecto deletéreo por los bajos niveles de PIWIL4. Dado que el CaPa es una enfermedad mortal y la supervivencia de los pacientes es bastante limitada, nuestros hallazgos permiten la identificación de dos subgrupos de riesgo diferentes que pueden manejarse clínicamente de forma independiente para mejorar la supervivencia. Sólo el tamaño del tumor mayor de 2 cm fue estadísticamente significativo junto con la baja expresión de PIWIL4 en el análisis multivariado de Cox para la supervivencia libre de progresión. Este resultado podría esperarse, ya que el tamaño del tumor en el diagnóstico está estrechamente relacionado con la supervivencia. Este hallazgo está apoyado por la literatura que muestra como la tasa de supervivencia a 5 años es de alrededor del 50% cuando los tumores están por debajo de 2 cm y cerca del 100% cuando los tumores están por debajo de 1 cm(4). Además, encontramos que un mayor porcentaje de pacientes con baja expresión de PIWIL4 presentan una mayor relación con el estadio T3 e invasión neural, en comparación con aquellos con alta expresión de PIWIL4.

7. La baja expresión de UNR se asocia con un mal pronóstico clínico en pacientes con cáncer de páncreas.

UNR es una RBPs relacionada con múltiples procesos; con respecto al cáncer, UNR se ha considerado un factor pro-oncogénico por su papel en la estabilización de

ciertos ARNm como el de c-fos y estimular la traducción del ARNm de c-myc(89), así como promover la metástasis de melanoma(88). Sin embargo, la sobreexpresión de UNR no siempre está asociada a la progresión tumoral, lo que indica que el papel preciso de la UNR en el cáncer depende del contexto o del ARNm al que esté regulando en ese momento. Por ejemplo, la sobreexpresión del oncogén HEPSIN en cáncer de próstata regula negativamente la expresión y la actividad IRES de UNR(90).

Para investigar la relación entre UNR y el CaPa, analizamos la supervivencia según la expresión de UNR en muestras de CaPa por inmunohistoquímica. Aquí, describimos por primera vez una asociación entre los bajos niveles de UNR y el mal pronóstico clínico de los pacientes con este cáncer. Ello nos hizo preguntarnos, si ocurriría lo mismo a nivel de ARNm. Entonces, analizamos los perfiles de expresión de dos bases de datos de pacientes independientes con perfil de expresión de ARNm, y los resultados de expresión de csde1 seguían el mismo patrón que los observados en nuestra serie midiendo los niveles de proteína. Estos resultados están en línea con los encontrados por Cornelis *et al.* que sugieren que una alta expresión constitutiva de UNR se vuelve citotóxica y puede conducir a la muerte celular(119). Es por ello por lo que en ciertos tipos de cáncer, la expresión de UNR puede actuar para suprimir la formación de tumores, como puede ser en el CaPa.

8. La expresión de UNR se asoció con el fenotipo inmunogénico del cáncer pancreático

Debido a que la baja expresión de UNR se asocia a un mal pronóstico clínico de los pacientes evaluados, lo que ocurre similarmente a las proteínas de PIWI, nos interesó conocer si UNR también se asocia con algún subtipo molecular de CaPa. Por ello, llevamos a cabo una correlación entre csde1 y los genes más significativos de cada subtipo molecular a nivel de RNAm.

El perfil de expresión disponible de una base de datos pública con 186 pacientes con CaPa procedente de la TGCA nos permitió llevar a cabo estos análisis. En este análisis, csde1 presentó una correlación moderada con los genes implicados en la vía de señalización del receptor de Toll (TCR, *Toll-cell receptor*). Esta vía regula la inmunidad innata y desencadena cascadas de señalización proinflamatorias(120). La

correlación entre la expresión de los ARNm de csde1 y tlr4, tlr7 o tlr8 sugiere que los pacientes con CaPa con alta expresión de UNR pueden presentar un fenotipo tumoral menos agresivo y más susceptible de ser eliminado por la respuesta inmune(112).

Los loci de *CDSE1* y *NRAS* se encuentran muy juntos en el genoma, con una distancia intergénica de sólo 150 nucleótidos. Esta ubicación especial planteó la posibilidad de interferencia transcripcional entre ambos genes. De hecho, dicha interferencia se encontró en tejidos de ratones, donde la eliminación del promotor de *CSDE1* condujo a un aumento en la acumulación de ARNm de NRAS(90). Contrariamente a los resultados en el ratón, no encontramos esta evidencia en tumores humanos, pero sí encontramos una correlación directa entre los niveles de ARNm de csde1 y nras en muestras de CaPa utilizando la base de datos de la TGCA. Pero esta correlación no se mantiene a nivel de proteína, ya que no encontramos relación entre los niveles de proteína de UNR y NRAS por inmunohistoquímica. Por lo tanto, la función protectora de UNR/*CSDE1* en CaPa no se explica por la baja regulación de NRAS, y debe depender de otros factores. Nuestras futuras investigaciones estarán dirigidas hacia la identificación de estos factores.

Valoración global de los resultados obtenidos

El trabajo de investigación llevado a cabo durante el desarrollo de esta tesis doctoral ha permitido estudiar en mayor profundidad la función y el papel pronóstico de las proteínas de unión a RNA, PIWI y UNR, en CaPa.

Aunque algunos estudios demostraron que estas proteínas presentaron características oncogénicas mediante diferentes mecanismos moleculares, sus funciones e impacto clínico en el CaPa han sido descubiertos a lo largo de esta Tesis Doctoral por primera vez. El papel de las proteínas PIWI en la tumorigénesis es bastante controvertido. A nosotros nos llamó la atención en particular el hecho de que PIWIL3 y PIWIL4 se expresaran en tejidos pancreáticos normales; por lo que nuestra hipótesis de que podrían ser oncogenes se encontró sin fundamento y simplemente se descartó .

Por otro lado, el hecho que los bajos niveles de PIWIL4 estuvieran relacionados con una reducida motilidad celular parece ir en contra de nuestros resultados que lo sugieren como un biomarcador de CaPa de mal pronóstico. Sin embargo, nuestros resultados sugieren que la falta de PIWIL4 podría aumentar la toxicidad del tratamiento y los posibles efectos adversos para los pacientes; pero también, una reparación defectuosa del tejido impulsada por un retraso en la motilidad celular a través de la reversión de la TEM y un defecto en la diferenciación celular. Todos estos mecanismos podrían retrasar el proceso de curación de los pacientes con CaPa y conducir a una supervivencia libre de progresión y supervivencia global más corta.

Después de la transcripción, los ARN siempre se asocian con proteínas de unión a ARN (RBP) para realizar actividades biológicas. Las RBP pueden interactuar con los ARN de manera dependiente de la secuencia y la estructura a través de sus dominios únicos de unión a ARN. En el desarrollo y la progresión de la carcinogénesis, las RBP están desreguladas de manera aberrante en muchos cánceres humanos con diversos mecanismos, como la alteración genética, los cambios epigenéticos, la regulación mediada por ARN no codificante y las modificaciones post-traduccionales(93).

La evidencia acumulada ha demostrado que los factores PIWI funcionan en la biogénesis de los piRNA y se asocian con los piRNA maduros para formar el complejo silenciador inducido por piRNA (piRISC) en la línea germinal, que protege la integridad del genoma silenciando elementos transponibles(40). No sólo las proteínas PIWI desempeñan un papel como factores promotores de la invasión, sino también sus piRNA asociados. Se ha descrito cómo la regulación negativa de piRNA-36712 promueve la invasión y migración de células tumorales; por lo tanto, se considera un supresor tumoral potencial en el cáncer de mama(121). Otro estudio apoya las propiedades supresoras de tumores de piR-823 porque su regulación positiva inhibe el crecimiento de células tumorales en modelos de cáncer gástrico(69). Otros experimentos funcionales han demostrado que piR-651 promueve la formación de tumores en el cáncer de pulmón de células no pequeñas mediado por Ciclina D1 y CDK4(56).

Por otra parte, la expresión de PIWIL1 no se asoció al pronóstico del CaPa, por lo que, se necesita más investigación para analizar el papel de PIWIL1 en la progresión de este tipo de tumor. Los resultados presentados aquí respaldan el papel de la expresión de la proteína PIWIL2 como un biomarcador pronóstico en el CaPa, y sugieren un vínculo entre la expresión de PIWIL2 y el subtipo molecular progenitor. Sin embargo, la función de PIWIL2 en el inicio y desarrollo del cáncer es bastante controvertida y no está clara. Por lo tanto, la investigación traslacional futura podría centrarse en aquellos piRNA regulados por PIWIL2. La identificación de los piRNA, tanto en tumores sólidos como en muestras de suero, y el descubrimiento de su función proporcionarán nuevos conocimientos sobre las proteínas PIWI y su papel como biomarcadores de diagnóstico, pronóstico o predictivos de respuesta.

Finalmente, en este estudio hemos mostrado que la expresión de PIWIL4 confiere quimiresistencia a través del factor HNF4A y que su desregulación *in vitro* frena la motilidad celular afectando el mecanismo de transición epitelio-mesenquima. Los futuros experimentos al respecto podrían estar dirigidos para evaluar estas mismas propiedades en modelos *in vivo*.

CONCLUSIONES

Las conclusiones derivadas de este trabajo son las siguientes:

1. La expresión de las proteínas PIWIL2 y PIWIL4 son factores asociados a mejor pronóstico en cáncer de páncreas capaces de predecir una supervivencia libre de progresión y supervivencia global más larga.

2. La expresión de PIWIL1 y PIWIL2 se asociaron al subtipo molecular progenitor de cáncer pancreático tanto a nivel de ARNm como de proteína.

3. La desregulación de PIWIL3 y/o PIWIL4 frena la motilidad celular *in vitro* afectando el mecanismo de transición epitelio-mesenquima tanto en las células tumorales como en las no transformadas.

4. PIWIL3 y PIWIL4 son factores cruciales en el mantenimiento de las células madre y en la resistencia a anoikis tanto en las células tumorales como en las pancreáticas sanas.

5. La expresión de PIWIL4 confiere quimiresistencia a través del factor HNF4A en cáncer de páncreas.

6. La baja expresión de UNR se asocia a mal pronóstico en los pacientes con cáncer de páncreas.

7. La expresión de UNR se asoció con el subtipo molecular inmunogénico del cáncer pancreático.

8. La expresión protéica de UNR es un potencial biomarcador pronóstico independiente en el cáncer de páncreas.

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ANEXO

Otras publicaciones y colaboraciones llevadas a cabo durante la Tesis Doctoral:

- KRAS and BRAF Mutations as Prognostic and Predictive Biomarkers for Standard Chemotherapy Response in Metastatic Colorectal Cancer: A Single Institutional Study. Garcia-Carbonero N, Martinez-Useros J, **Li W**, Orta A, Perez N, Carames C, et al. Cells. 2020 Jan 15;9(1).

- UNR/CSDE1 Expression Is Critical to Maintain Invasive Phenotype of Colorectal Cancer through Regulation of c-MYC and Epithelial-to-Mesenchymal Transition. Martinez-Useros J, Garcia-Carbonero N, **Li W**, Fernandez-Aceñero MJ, Cristobal I, Rincon R, Rodriguez-Remirez M, Borrero-Palacios A, Garcia-Foncillas J. J Clin Med. 2019 Apr 25;8(4).

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- Oxidative Stress: A New Target for Pancreatic Cancer Prognosis and Treatment. Martinez-Useros J, **Li W**, Cabeza-Morales M, Garcia-Foncillas J. J Clin Med. 2017 Mar 9;6(3).